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HISTOCHEMICAL REACTIONS OF THE ENTEROCHROMAFFIN CELLS AND THE 5 HYDROXYTRYPTAMINE CONTENT OF THE MAMMALIAN DUODENUM

BY

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Kirjapaino

PREFACE

The subject of the present study was proposed in 1964 by Professor Olavi Eranko M D Ph D Head of the Department of Anatomy University of Helsinki This work is a part of the departmental research project on the histo chemistry of biogenic amines

I wish to express gratitude to Professor Eranko for his encouragement continued support and valuable criticism from the first experiments to the preparation of the manuscript

Professor Matti Paasonen M D read the manuscript and suggested significant alterations for which I am very greatful to him

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Miss Elvi Kaukokallio corrected the English

Finally I wish to express my warmest thanks to my wife Sisko Penttila M A for all her help and correction of the English manuscript

Helsinki July 1966

Antti Penttila

CONTENTS

INTRODUCTION

Small cells which stained specifically brown after fixation with solutions containing dichromate were first described in the stomach of the dog and rabbit by Heidenhain in 1870 This kind of cells later called enterochromaffin cells were found by Toldt (1880) also in other species Nicolas (1891) was the first to find enterochromaffin cells in the intestine. He was convinced that the entero chromaffin cells in the lizard were cells *sui generis* Later it was generally stated by many other investigators that the enterochromaffin cells in the alimentary canal were a special cell type, although Ellenberger (1911) denied that they were autonomic cells and Eklof (1914) considered the enterochromaffin granules arti facts caused by an unsuccessful fixation

In the literature enterochromaffin cells have many different names Earlier they were often called after their investigators the Kultschitzky Schmidt Ciaccio or Masson cells Because of the granular cyto plasm Kaufmann Wolf (1911) introduced the term basal granular cells Mostly however the cells were called after their staining properties Schmidt (1905) introduced the term yellow cells Kull (1925) chromaffin cells and Cordier (1926) chromo argentaffin cells The terms argent affin, first used by Masson (1914) argyrophil (Hamperl 1932) and argentophil cells (Erspamer 1937) indicate the importance of silver reactions for demonstrating these cells Ciaccio (1906 1907) found that the staining properties of the chromaffin cells of the gut were similar to those of the medullary cells in the adrenal gland already commonly called chromaffin cells and introduced the term enterochromaffin cells This term has been generally accepted and it is universally used today

The histochemical properties of the enterochromaffin cells are quite dissimilar to those of the adjacent epithelial cells in the alimentary canal This dissimilarity has given reason for many theories about the chemical structure of the entero-chromaffin substance (Ciaccio 1907 Hamperl 1925 Cordier and Lison 1930 Gomori 1948) It was suggested by Erspamer and his associates (Erspamer 1940a Vialli and Erspamer 1940 Asero *et al* 1952 Erspamer and Asero 1952a b) that 5-hydroxytryptamine is the specific substance in the enterochromaffin cells This theory has been supported by many comparative and experimental inves tigations

However there are several unsolved problems concerning the enterochrom affin cells and the intestinal 5-HT as will become apparent from the following literature review Therefore the present study was undertaken to elucidate the enterochromaffin cell system in different mammals and its correlation to 5 hy droxytryptamine

INTRODUCTION

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However there are several unsolved problems concerning [illegible] affin cells and the intestinal 5 HT, as will become apparent [illegible] enterochromaffin literature review. Therefore the present study was undertaken [illegible] following enterochromaffin cell system in different mammals and [illegible] droxytryptamine.

REVIEW OF LITERATURE

THE ENTEROCHROMAFFIN SYSTEM

In a strict sense the enterochromaffin system comprises the enterochromaffin cells (henceforth to be referred to as EC) of the gastrointestinal tract and of all glandular elements of intestinal origin (Erspamer 1958) Earlier it was generally accepted that the EC were morphologically and chemically identical in all the intestinal parts of different animal species Therefore it was assumed that also the staining properties of the EC were similar Clara (1933a b) however showed remarkable differences in various staining reactions between EC in different vertebrate classes With the aid of two silver reactions Erspamer (1937 1938) and Vialli and Erspamer (1939) divided the cells of the enterochromaffin system into four types 1) pre-enterochromaffin non argyrophil cell 2) pre-enterochromaffin argyrophil cell 3) enterochromaffin cell proper 4) empty enterochromaffin cell This classification represented various successive stages in the maturation of the EC. True EC could be stained with all the specific staining reactions such as argyrophil argentaffin ferric ferricyanide diazo coupling and indophenol reactions On the other hand the argyrophil cells stained specifically only by the silver impregnation method in which reaction the extraneous reducing substance caused the blackening of the EC. In the argentaffin reaction the EC themselves were able to reduce the silver solutions The first and last stages of the cycle could not be defined histochemically

The true EC were stated to contain a specific substance 5-hydroxytryptamine whereas only its precursors were found in the argyrophil stage (Erspamer 1958) This theory was supported by Monesi (1960a) who demonstrated that the argyrophil cells preceded EC in the intestine of the chick embryo It was noted by Hellweg (1952) and Hamperl (1952) that among the human intestinal EC there were 50 % argyrophil and 5 % argentaffin cells and 45 % EC showing both staining properties With adequate methods Singh (1963 1964a) could find in the human intestine no EC with argentaffin properties only

This confirmed Erspamer s theory that there were only two histochemically demonstrable types of EC. The difference between argyrophil and argentaffin cells was demonstrated in the electron microscopy by Toner (1964) The granules of the argentaffin cells varied more in shape and size and they were larger and coarser than the granules in the argyrophil cells Ratzenhofer and Leb (1965) further showed that the argentaffin granules were osmiophilic but the argyrophil granules osmiophobic.

The concept of the enterochromaffin system was expanded by Erspamer (1958 1961) He called EC all such cells also the cytoplasmic granules of which could be stained after the formalin fixation with argentaffin chromaffin diazo coupling and alkaline thioindoxyl reactions and that showed a formalin induced fluorescence Cells of this kind were found in the mammalian urethra

birds (Quaroni 1956) and reptiles (Vialli and Casati 1958) in the cutaneous glands of amphibians (Vialli 1955) in the lizard oviduct and the frog urinary bladder (Erspamer 1958) in the venous glands of scorpions (Adam and Weiss 1959) in the posterior salivary glands of octopods and in the hypobranchial body of some molluscs (Erspamer 1958) and in the nematocysts of some coelenterates (Hamon 1955) All the cells of the enterochromaffin system contained 5-hydroxy tryptamine or related substances and therefore Erspamer included also the mast cells of the rat and mouse into this system

On the other hand Feyrter (1953) included the EC of the intestinal tract in another system the so called »helle Zellen« organs Also this classification was introduced on the ground of the staining properties of EC granules The scattered clear cells were derived from the inner and outer epithelium of the body and apparently had an endocrine function

In the present study the EC in the intestinal tract will be mainly discussed

ENTEROCHROMAFFIN CELLS IN DIFFERENT SPECIES

EC are to be found in the alimentary canal of all the classes of vertebrates investigated and also in some groups of invertebrates (Erspamer 1958)

Most studies have been made with mammals In adult man the EC were first described by Zimmermann (1898) and in the nursling by Bloch (1903) Kultschitzky (1897) and Moller (1899) discovered EC in both the small and the large intestine of some domestic animals In all the adult mammals investigated the EC were found in every part of the intestinal tract from the stomach to the anus (Muthmann 1913 Tang 1922 Kull 1925 Hamperl 1925 Cordier 1926 Tehver 1930 Clara 1933a Jacobson 1939 Dawson 1945) Studies on embryos are not so numerous but they all show that EC are to be found already during embryonic life In the human intestinal tract the EC appear between the fifth and sixth month of gestation according to Kull (1925) and between the sixth and twelfth week according to Cordier (1926) and Cole and McKalen (1962) In the intestine of domestic animals the EC were found during the first half of gestation (Tehver 1930) Faustini (1955) demonstrated EC in the fifth week of intrauterine life in the calf embryo

The existence of EC in the intestinal tract of most species of birds has been stated by Muthmann (1913) Kull (1925) and Clara (1926a b) In chick embryos of 14—16 days Monesi (1960a b) found intestinal EC both in the normal and tissue cultured intestine In the reptilia and amphibia the EC were found almost in every species investigated (Kull 1925 Cordier 1926 Vialli 1929 Toro 1930 Clara 1932 1933a) Only some species of fish showed EC (Kull 1925 Cordier 1926 Rogosina 1928 1930) De Filippi (1930) found no EC in the teleosts but recently the existence of the argyrophil cells in this fish group was demonstrated by Erspamer (1958)

DISTRIBUTION OF THE ENTEROCHROMAFFIN CELLS

According to Schmidt (1905) and Cordier (1926) the distribution of the EC was quite even in different parts of the intestine However Hamperl (1925) reported that EC were most numerous in the duodenum and that there was a steady decrease in their number in the caudal direction of the intestine This observation was later confirmed by precise quantitative estimations made by Tehver (1930) in the pig horse dog and cat by Hoeschen (1937) in the guinea pig and by Munch (1939) in the rat It was noted by Hoeschen (1937) Vetter (1937 1938) and Munch (1939) that in the guinea pig and rat EC were most numerous in the duodenal part near the pylorys and that their count strikingly decreased already in this part of the small intestine in the anal direction Only in the cow were large numbers of EC found in the rectum (Tehver 1930) In the

coecum of some mammals EC were relatively numerous (Kull 1925 Cordier 1926 Tehver 1930) Also in the human appendix the number of EC was considerably high (Sprafke 1927 Masson 1928 Schack 1932)

In other vertebrates Kull (1925) and Clara (1926a) observed a similar duodenal predominance in the distribution of the EC except for some bird and fish species which showed an increasing number of EC in the caudal direction

In the stomach the demonstration of the EC in several species was difficult before the argyrophil reaction was used Dawson (1944 1945) and Sharples (1945a b) showed that EC were rather numerous also in the stomach of mammals especially in the fundic region In the oesophagus Kull (1925) and Feyrter (1934) found very few EC situated always near the cardia but according to Tehver (1930) Dawson (1945) and Sharples (1945a) the EC were absent in the oesophagus as also in the squamous stratified epithelium of the stomach

It has been generally observed that even in the same species the individual variations in the EC count in the intestinal tract are great Hallier (1937) Klemm (1937) Vetter (1938) and Schumann (1939) showed that the number of EC was distinctly greater in adult female guinea pigs rats and mice than in male animals Schumann further observed a steady increase of the EC count in the guinea pig during gestation Since the time of Heidenhain (1870) it has often been reported that the amount and kind of food have a great influence on the number of stained EC

In the alimentary canal of all vertebrates the EC were diffusely distributed among the epithelial cells (Clara 1933a) Sometimes great EC accumulations were observed The most numerous EC were found in the mucosa particularly in the basal parts Also in Brunner s glands some EC were often seen

MORPHOLOGY OF THE ENTEROCHROMAFFIN CELLS

The coarse cytoplasmic granularity of the EC was observed already by Heidenhain (1870) This property made EC easily distinguishable from the other gastrointestinal cells According to Hamperl (1925) the average size of the EC was 21—24 micra in length and the cell basis was 8—12 micra in breadth even though there were variations in different animals Also the shape of the EC varied considerably in the different parts of the mucosa

Electron microscopy studies on mammalian EC have been recently made by Christie (1955) Helander (1961) and Wetzstein *et al* (1962) They all observed that the cytoplasm of the EC contained numerous more or less evenly dispersed oval or circular bodies The specific granules surrounded by a membrane had an irregular internal structure Their average size was 0 2—0 3 micra The granules were mostly situated in the basal part of the EC and were often absent in the apical part The endoplasmic reticulum was well developed particularly above the nucleus The EC were in direct contact with the basal membrane and in some cells the ciliated free cytoplasm extended to the intestinal lumen

HISTOCHEMICAL REACTIONS OF ENTEROCHROMAFFIN CELLS

Fixation of Enterochromaffin Cells

Already in the early studies formalin was generally used for the fixation of EC. Hamperl (1925 1927) showed that formalin was necessary for the preservation of the staining properties of EC. Good results were obtained with pure aqueous solutions of formalin or with Bouin s or Orth s fluids In different species the optimum composition of the fixative has been found

to vary considerably Clara (1933b) demonstrated that Bouin s fluid was especially effective in birds but not so good in amphibia After 5-hydroxytryptamine (henceforth to be referred to as 5 HT) was found in the EC Barter and Pearse (1953) and later Holcenberg and Benditt (1961) showed that synthetic 5 HT and formaldehyde condensed to an unconjugated tetrahydro-4-carboline derivative This compound showed an intense yellow fluorescence in ultraviolet light The dry formaldehyde vapour also condensed with 5-HT to this unsaturated derivative (Corrodi and Hillarp 1963) Holcenberg and Benditt (1961) demonstrated that the carboline derivative was highly insoluble in water and in various organic solvents but dissolved readily in 0 1 N HCl This derivative is the reason for the specific staining properties of the fixed EC.

It was found quite early that fixatives containing alcohol acids or sublimate fixed EC less effectively (Hamperl 1925) Using paper chromatography Eder *et al* (1962) demonstrated that 5 HT and formaldehyde produced no condensation derivative in acids and no water insoluble derivatives in alcoholic solutions In neutral water solutions four condensation products were formed with different chromatographic properties In striking contrast to all the other specific staining reactions in the EC the argyrophil reaction was positive also in tissues fixed in fixatives containing alcohol (Dawson 1944 Hellweg 1952 Hamperl 1952 Eder *et al* 1962) Eder *et al* further showed that also the argyrophil reaction was negative in EC fixed in pure alcohol

Earlier it was generally assumed that the granules in the EC were not original but artifacts produced by formalin fixation This hypothesis was denied by Christie (1955) He demonstrated that the dark EC granules fixed in an osmium tetroxide solution without any formalin treatment were beautifully seen in electron microscopy

Fluorescence Reaction

Eros (1932) was the first to find golden yellow fluorescent cells in the intestine and some endocrine glands fixed in a formalin solution Only formalin made this fluorescence possible but the nature of the fluorogenic material remained obscure Jacobson (1939) measured the fluorescence spectra in human carcinoid tumour cells He also studied the influence of temperature oxidation alkaline solutions enzymes and illumination on the properties of the fluorescence In general this 5 HT fluorescence was very permanent Eranko (1951 1952) was the first to observe that some medullary cells in the adrenal gland showed a strong greenish fluorescence in formalin fixed sections which was later (Eranko 1955a) shown to be due to noradrenaline Shepherd *et al* (1953) studied several amines by chromatography after formalin treatment and found that the fluorescence characteristics of synthetic 5 HT bufotenine and bufotenidine were quite similar It was noticed by Barter and Pearse (1953 1955) that the fluorescence of synthetic 5 HT and that of the EC in the guinea pig was of a similar yellow colour They first treated the freeze dried tissues in damp formaldehyde vapour but the method was not very successful because of the easy diffusion of 5 HT Lagunoff *et al* (1961) and Eranko (1961) noticed that the dry formaldehyde vapour converted several monoamines into strongly fluorescent compounds Based on a similar observation Falck and Torp (1961) developed the method suitable for the distinct histochemical demonstration of fluorescent monoamines in freeze dried tissues

The mechanism of the formaldehyde induced fluorescence reaction has been recently studied thoroughly particularly by using model systems and the chemical as well as the histochemical background is now well understood (Falck *et al* 1962 Corrodi and Hillarp 1963 1964 Corrodi *et al* 1964 Corrodi and Jonsson 1965) The catecholamines were converted into isoquinoline derivatives which had a green fluorescence colour sometimes a yellowish tinge 5 HT and 5-hydroxytryptophan reacted principally in the same way Only those indolic compounds with a tryptamine side chain and with an unsubstituted 2 position are condensed with formaldehyde

(Falck and Owman 1965) Only the indoles with 5-hydroxy and 5-methoxy groups formed condensation products which showed an intensive yellow fluorescence In the activation spectra the main peaks for catecholamines and indole derivatives were in the same region whereas the main emission peaks were at 480 nm for catecholamines and at 510 nm for 5 HT This difference between the emission peaks made it possible to distinguish the fluorescence of the catecholamines from that of the indolic derivatives With this method Bertler *et al* (1964) obtained a very strong fluorescence of 5 HT in the pineal gland of the rat Also very small amounts of 5-HT in the brain stem were demonstrated with this method (Dahlstrom and Fuxe 1964) Lagunoff *et al* (1961) found in mast cells of the rat tongue a strong yellow fluorescence after paraformaldehyde treatment The uptake of several monoamines by mesenteric mast cells of the mouse was beautifully demonstrated by fluorescence microscopy (Eranko and Kauko 1965) Only Norberg (1964) and Hammarstrom *et al* (1966) have shown that the EC exhibit a strong yellow fluorescence in the small intestine of the cat mouse and rat

Silver Reactions

Argentaffin Reaction — Masson (1914) was the first to observe that Fontana s ammoniacal silver solution stained the EC blackish in the human intestine after formalin fixation The method was simplified by Hamperl (1925) This »Masson Hamperl s method» has been the most commonly employed one for the demonstration of the EC Modifications of the argentaffin reaction were introduced by Gomori (1948) and Burtner and Lillie (1949) but the results obtained were essentially similar to those obtained with the original method

In the argentaffin reaction the formalin fixed EC themselves were capable of reducing ammoniacal silver solutions into metallic silver (Hamperl 1925) Lison (1931) observed the low chemical specificity of this reaction He pointed out that it was given positive by several biological substances among which phenols were the most important According to Glick (1949) also many other reducing substances made the reaction strongly positive Pearse (1953) considered the argentaffin reaction specific for aldehydes which opinion however was contradicted by Lillie (1954b)

Argyrophil Reaction — In silver impregnation methods the precipitate is produced by an extraneous reducing agent The precipitate is first formed in »argyrophil» sites of a tissue. Such silver impregnation methods have mainly been used for the staining of nervous tissue but Hasegawa (1923) developed a modification of his own and showed that the EC in the human small intestine and appendix had argyrophil properties Thereafter many other modifications designed for nervous tissues have been used for the demonstration of the EC, such as Agduhr s method (Danisch 1924) Bielschowsky s method (Hamperl 1925) Masson s method (Masson 1928) Gros Schultze s method (Hamperl 1932) or Singh s (1964b) method All these methods especially those in which large tissue pieces are impregnated were often reported to be capricious Bodian (1936 1937) developed a more reproducible method Instead of the silver nitrate solution the organically bound silver compound »Protargol S» was used Dawson and Barnett (1944) found this method particularly suitable for the demonstration of the EC Nowadays this method is most commonly used for the staining of the »argyrophil» cells

Hamperl (1925) already pointed out the principal difference between the argentaffin and argyrophil reactions The reducing ability of the enterochromaffin substance makes the EC directly positive in the argentaffin reaction but why the argyrophil reaction becomes positive only after the addition of an extra reducer is obscure. Although the silver impregnation method was chemically highly unspecific, it was selective for many morphological structures (Gomori 1948)

Diazo Coupling Reaction

Cordier and Lison (1930) were the first to use fresh diazo [illegible] for the [illegible] of the EC granules Clara (1932 1934) obtained similar results with both fresh and stable [illegible] Lison (1931) and Clara (1932) further observed that the EC reacted [illegible] It was noted by Pearse (1960) and Lillie *et al* (1961) that some EC were [illegible] 4 0—5 2 The pH optimum was however clearly on the alkaline side The [illegible] specifically stained organelles from the background depended on the [illegible] Lillie *et al* (1961) demonstrated that the best results were [illegible] salts Also many stable nitroanilines nitroanisidines, and [illegible]

The chemical basis of the diazo coupling reaction was studied by Cordier and Lison [illegible] The reaction was given by aromatic amines at acid pH and by [illegible] lic hydroxyl group must be unsubstituted. At least one of the [illegible] xyl group must also be free The reaction [illegible] the EC [illegible] and depended on the structure of the salts and reacting substances

Indophenol Reaction

Gibbs (1927) found that phenols reacted with 2 [illegible] buffer solution The reaction product was an indophenol [illegible] pended on the reacting phenol Gomori (1948) was the first [illegible] try According to him the reaction was specific for [illegible] for catechols and *para* substituted phenols The Gibbs reaction has seldom been used for demonstrating EC. It was shown by Lillie and Glenner (19[illegible]) that the reaction was less [illegible] in the EC and carcinoid tumours Moderately [illegible] by Barter *et al* (1957) in the duodenum and stomach of the rabbit and [illegible] appendix and by Gerzeli (1961) in the intestine of an invertebrate [illegible]

Lison (1931) used a dimethyl *p*-phenylenediamine salt as a [illegible] the presence of an oxidizing agent In the vertebrate EC this reaction produces [illegible] and is therefore not commonly used

Ferric Ferricyanide Reaction

The ferric ferricyanide reaction was introduced by Golodetz and Unna (1909) into histochemistry Ferricyanide was reduced to ferrocyanide by tissue components and thereafter it was converted in the presence of ferric ions to insoluble Prussian blue [illegible] Gomori (1948) first noticed that this reaction stained also the EC granules intensively [illegible] (1954) found that the reaction mixture containing ferric chloride and potassium ferricyanide in a molar proportion of 15 1 in contrast to the earlier 6 1 stained the EC more effectively

The ferric ferricyanide reaction is chemically quite unspecific and all reducing substances give a positive reaction (Lillie and Burtner 1953 Lillie 1954)

Ferric Chloride Reaction

It was demonstrated by Cordier and Lison (1930) that all [illegible] phenols reacted in a dilute ferric chloride solution The method is seldom used for the demonstration of the EC granules because of its weak staining ability Therefore Cordier and Lison did not dare to speak of a positive reaction in the EC. However Clara (1933a b) observed that the EC in mammals and birds turned greenish whereas in reptilia and amphibia they [illegible]

Ninhydrin Reaction

Jepson and Stevens (1953) discovered a specific test for the tryptamines which did not contain substituents either at position 2 or at the nitrogen atom. In ultraviolet light synthetic compounds of this kind gave on dried paper a strongly blue green fluorescence after treatment with ninhydrin in acetone containing glacial acetic acid Holcenberg and Benditt (1959 1961) introduced this principle into histochemistry They found that after formalin fixed sections of the guinea pig duodenum had been treated in the ninhydrin vapour in vacuum the EC granules were stained orange red This colour was given only by indolethylamine compounds On the indole ring there ought to be a free 2 position a free α-carbon atom as well as an amine group on the ethylamine side-chain The compounds of the tetrahydro-4-carboline derivatives with unsubstituted 3- 4- and 5 positions and substituted pyridic ring gave a positive reaction The sensitivity of the reaction was similar to that of the argentaffin and diazo coupling reactions. According to Barka and Anderson (1963) the ninhydrin reaction is one of the most specific for the enterochromaffin substance

Oxidative Agents

Heidenhain (1870) observed that the unfixed EC granules turned yellow in a potassium dichromate solution Later it was demonstrated that many other oxidizing agents such as nitric acid bromine water potassium permanganate and iodates produced a yellow colour in the EC (Patzelt 1936 Gomori 1948) Recently it was noted by Eriksen *et al* (1960) and by Smuckler *et al* (1960) that synthetic 5 HT was converted with the aid of oxidants to polymers and brownish pigments The polymers were formed by dehydrogenative coupling through the aromatic rings of the molecule They further suggested that obviously also the enterochromaffin substance was converted by oxidizing agents to polymers rather than to the quinhydrone type of substances as postulated by Gérard *et al* (1930)

Other Reactions

Clara (1935) studied the chemical nature of several usual dyes which stained the EC granules These dyes contained in their molecule hydroxyl groups arranged in a catechol manner In dilute solutions of haematoxylin gallocyanin gallamine blue celestin blue alizarin cyanin R or naphthazarin the EC granules were stained quite specifically According to Clara the dyes were absorbed by the EC granules In contrast to this Lillie (1961) suggested that the reaction was given positive by an open phenolic group in the enterochromaffin substance He found no reaction in tryptamine models

Quastel s reagent (containing ammonium molybdate and glacial acetic acid) for demonstrating *o*-dioxybenzene derivatives was shown by Clara (1933a b) to be positive in the EC of mammals and birds and negative in those of reptilia and amphibia

The chemistry of Mallory Heidenhain s azocarmine staining reaction is still unknown Gomori (1946 1948) demonstrated that also this reaction specifically stained the EC granules red or purplish especially in human carcinoid tumours

It was demonstrated by Christie (1954) that the EC granules gave a positive acid haematein reaction Pearse (1960) however showed that the reaction was weak in the EC of man and guinea pig Besides all the other reactions for phospholipids were negative in the EC

Pearse (1956) found that the formalin fixed EC were stained reddish brown in the alkaline thioindoxyl reaction The β-carboline derivative of 5-HT and formaldehyde was oxidized with iodine to the quinoneimine This derivative reacted in an alkaline solution with unsubstituted or substituted thioindoxyls forming thioindigoid dyes

CHEMICAL CHARACTER OF THE ENTEROCHROMAFFIN GRANULES

The staining reactions described have caused many theories concerning the chemical nature of the enterochromaffin granules

Catechol Theory

Ciaccio (1907) demonstrating that the EC and adrenal medullary cells were stained quite similarly yellow in dichromate solutions suggested that adrenaline was the principal substance of the EC This hypothesis was supported by Masson s (1914) experiments with the argentaffin reaction Hamperl (1925) showed that the silver and chromaffin reactions were caused by a brenzcathecin derivative This theory was supported by several other investigations (Cordier and Lison 1930 Lison 1931 Clara 1932 1933a 1934 Clara and Canal 1932 Vialli and Erspamer 1933) It was demonstrated that the diazo coupling and indophenol reactions were given by phenols in alkaline solutions The monophenols and polyphenols were excluded by chemical experiments Earlier Verne (1923) had shown that only *para* and *ortho*diphenols were capable of giving a positive chromaffin reaction Positive ferric chloride and Quastel reactions suggested that the enterochromaffin substance ought to be an *ortho*diphenol This suggested catechol substance an *o*-dihydroxybenzene derivative had a simple unknown side-chain in the *para* position to a hydroxyl group

Pteridine Theory

From the argentaffin and diazo coupling but especially from the positive murexide reaction in the EC Jacobson (1939) concluded that the enterochromaffin granules contained a purine compound Jacobson s positive Molisch and Schiff reactions in the EC which observations it was not possible to confirm later (Lillie 1961) suggested that the enterochromaffin substance was a carbohydrate compound Jacobson made also spectrographic studies and observed the fluorescence properties to be quite similar in xanthopterin and in carcinoid enterochromaffin substance Based on these and other chemical comparisons Jacobson and Simpson (1946) and Jacobson (1954) claimed that the enterochromaffin substance was a pteridine compound This hypothesis was shown to be incorrect by Gomori (1948) and Pearse (1960)

Resorcinol Theory

Gomori (1948) showed that the catechol theory had three fallacies Catechols and their derivatives were more strongly reducing agents than the enterochromaffin substance Secondly the positive indophenol reaction was against a *para* substituted phenol Thirdly in the diazo coupling reaction there were many exceptions in the staining ability of *ortho-* and *para*diphenols Gomori compared the staining properties of various substances with those of the EC granules in model systems From the results obtained with argentaffin diazo coupling and Gibb reagents he concluded that the enterochromaffin substance was a derivative of *meta*diphenol (resorcinol) rather than an *ortho*diphenol as previously suggested Gomori s theory was supported by Lillie *et al* (1953) Recently the 5 HT theory has abolished the resorcinol theory although Gomori (1954) claimed that it was impossible to show if 5 hydroxyindole resorcinol or some unknown chemical compound was the main enterochromaffin substance in the diazo coupling reaction

5 Hydroxytryptamine Theory

The greatest advances in studies on the enterochromaffin system were made by Erspamer and his associates during the years 1940—1952 From the stomach mucosa of the rabbit Erspamer

Ninhydrin Reaction

Jepson and Stevens (1953) discovered a specific test for the tryptamines which did not contain substituents either at position 2 or at the nitrogen atom In ultraviolet light synthetic compounds of this kind gave on dried paper a strongly blue green fluorescence after treatment with ninhydrin in acetone containing glacial acetic acid Holcenberg and Benditt (1959 1961) introduced this principle into histochemistry They found that after formalin fixed sections of the guinea pig duodenum had been treated in the ninhydrin vapour in vacuum the EC granules were stained orange red This colour was given only by indolethylamine compounds On the indole ring there ought to be a free 2 position a free α-carbon atom as well as an amine group on the ethylamine side-chain The compounds of the tetrahydro-4-carboline derivatives with unsubstituted 3- 4- and 5-positions and substituted pyridic ring gave a positive reaction The sensitivity of the reaction was similar to that of the argentaffin and diazo coupling reactions According to Barka and Anderson (1963) the ninhydrin reaction is one of the most specific for the enterochromaffin substance

Oxidative Agents

Heidenhain (1870) observed that the unfixed EC granules turned yellow in a potassium dichromate solution Later it was demonstrated that many other oxidizing agents such as nitric acid bromine water potassium permanganate and iodates produced a yellow colour in the EC (Patzelt 1936 Gomori 1948) Recently it was noted by Eriksen *et al* (1960) and by Smuckler *et al* (1960) that synthetic 5-HT was converted with the aid of oxidants to polymers and brownish pigments The polymers were formed by dehydrogenative coupling through the aromatic rings of the molecule They further suggested that obviously also the enterochromaffin substance was converted by oxidizing agents to polymers rather than to the quinhydrone type of substances as postulated by Gérard *et al* (1930)

Other Reactions

Clara (1935) studied the chemical nature of several usual dyes which stained the EC granules These dyes contained in their molecule hydroxyl groups arranged in a catechol manner In dilute solutions of haematoxylin gallocyanin gallamine blue celestin blue alizarin cyanin R or naphthazarin the EC granules were stained quite specifically According to Clara the dyes were absorbed by the EC granules In contrast to this Lillie (1961) suggested that the reaction was given positive by an open phenolic group in the enterochromaffin substance He found no reaction in tryptamine models

Quastel s reagent (containing ammonium molybdate and glacial acetic acid) for demonstrating *o*-dioxybenzene derivatives was shown by Clara (1933a b) to be positive in the EC of mammals and birds and negative in those of reptilia and amphibia

The chemistry of Mallory Heidenhain s azocarmine staining reaction is still unknown Gomori (1946 1948) demonstrated that also this reaction specifically stained the EC granules red or purplish especially in human carcinoid tumours

It was demonstrated by Christie (1954) that the EC granules gave a positive acid haematein reaction Pearse (1960) however showed that the reaction was weak in the EC of man and guinea pig Besides all the other reactions for phospholipids were negative in the EC.

Pearse (1956) found that the formalin fixed EC were stained reddish brown in the alkaline thioindoxyl reaction The β-carboline derivative of 5 HT and formaldehyde was oxidized with iodine to the quinoneimine This derivative reacted in an alkaline solution with unsubstituted or substituted thioindoxyls forming thioindigoid dyes

PROBLEMS OF THE PRESENT STUDY

In spite of the numerous separate investigations concerning the histochemical properties of the EC, no systematic comparison has been made between various mammalian species. Moreover, the ferric ferricyanide, diazo coupling and indophenol reactions have seldom been used for the demonstration of EC. The formaldehyde induced fluorescence in the EC has been reported only as a by-observation. The hypothesis that the EC count and the 5-HT concentration are in a direct correlation to each other in the intestinal tract is based on the separate investigations made by anatomists and pharmacologists. Both variables greatly depend on the environmental and racial conditions of the animals, as well as on the techniques used for the quantitative determinations. In order to avoid uncertain estimations of the correlation between the EC and 5-HT *it is necessary to* determine both quantities in adjacent tissue pieces. This kind of determinations have hardly been made earlier.

The main problems of the present study were formulated as follows:

— Are the histochemical properties of the EC similar in the different mammals?

— How effective are the different reactions in demonstrating the mammalian EC?

— Are there one or several kinds of histochemically demonstrable EC?

— Is there a correlation between the number of EC and the 5-HT content in the normal mammalian duodenum?

— What is the effect of reserpine and cortisone on the EC and on the 5-HT content?

MATERIAL

The material consisted of the small intestines of 32 sheep 26 cows 26 pigs 18 horses 82 guinea pigs 19 mice 176 rats and 9 rabbits Only females were used

The sheep pigs and horses of Finnish domestic breed and the cows of Ayshire breed were obtained from the municipal slaughterhouse of Helsinki The guinea pigs and white rabbits of an unknown strain were bred at Minkkaa farm (Orion Oy) The white mice of an unknown strain and the white rats descendants of the Sprague Dawley strain were bred in the colony of the Department of Anatomy

The rats varied in age from 1—2 hours after birth to adult Nine of the guinea pigs were new born all the other animals were adult. The sheep were 2—4 the cows 3—10 the horses 5—15 and th rabbits 1—2 years old the pigs 6—8 and the mice 4—6 months the adult guinea pigs and rats 3—6 months of age

Before slaughtering the animals were kept in stables for 1—4 days at room temperature The sheep received 1 kg the cows 3 kg and the horses 5 kg of hay and the pigs 1 kg of oats per day The guinea pigs were allowed to eat hay swedes and »guinea pig pellets» (3.3 % raw fat 22 1 raw protein 7 0 % raw fibre 44 8 % nitrogen free extractable substances 9 1 % ash and 13 7 % water made by Orion Oy) The mice and rats were fed on a standard cube diet (3 5 % raw fat 24 0 raw protein 5 5 % raw fibre 43 5 % nitrog n free extractable substances 13 0 ash and 10 5 water made by Keskusosuusliike Hankkija) The rabbits were fed on hay oats and swedes All the animals were allowed to drink water *ad libitum* the laboratory animals had al o free access to food

The sheep were killed by a blow on the head the cows and horses by shooting in the head and the pigs by electrocution The laboratory animals were killed by a blow on the neck without anaesthesia The duodenal parts were removed in the slaughterhouse from the sheep within 2—5 from the cows and pigs within 10—15 and from the horses within 20 minutes after killing From the laboratory animals the tissue pieces were taken immediat ly after death

The intestinal pieces from the sheep cows p gs horses and rabbits were always taken near the pylorus The pieces were cut into two parts one for the freeze drying procedure and the usual staining reactions and one for the 5 HT determination From the guinea pigs mice and rats the proximal third of the duod num was removed The middle portion of this duodenal third was used for the freeze-drying procedure or the histochemical staining reactions the remaining parts for the 5 HT det rmination The pieces used in the histochemical reactions were immediately put into fixatives The pieces for the freeze drying procedure were cooled in liquid nitrogen kept in it for less than 2 hours and then transferred into a freeze drying apparatus or stored with solid carbon dioxide at —78 C for 1—7 days The duodenal pieces for the 5 HT determination were kept for 10—20 minutes in a thermos box at ±0 C

METHODS

QUALITATIVE METHODS

A solution of 80 % alcohol (90 ml) 99—100 % glacial acetic acid (5ml) and 37—40 % w/v neutral formaldehyde (5ml) was used as fixative for demonstrating the argyrophil reaction in the EC In all the other staining reactions the tissue pieces were fixed in 5 aqueous formalde hyde solution In both cases the fixing time varied from 2 to 5 days The fixed tissues were dehydrated in alcohol series and embedded through butanol in paraffin wax The sections were cut at 5 micra The stained sections were dehydrated in alcohol cleared in xylene and mounted in Canada balsam.

The following staining techniques were used

Argyrophil Reaction — Bodian s (1936) silver impregnation method was used The sections were immersed in 1 Protargol S (Sterling Drug Inc) at 37 C for 12—24 hours Copper nails were placed on the bottom of the staining dish As a reducing agent served a solution of hydroquinone (1 g) sodium sulphite (5 g) and distilled water (100 ml) The reducing time varied from 5 to 15 minutes The final staining was carried out in 1—2 % oxalic acid solution Overstaining was carefully avoided

Argentaffin Reaction — The Fontana solution was used (see Romeis 1948) The final solution had to be distinctly turbid and without the odour of ammonia The solution was used after 24 hours The staining procedure was carried out by the method of Hamperl (1925) for 1—3 days The stained sections were washed in distilled water and fixed in 5 % sodium thiosulphate solution for 1—2 minutes

Ferric Ferricyanide Reaction — The reduction techniques described by Lillie (1954a) and Pearse (1960) were used The latter technique was mainly followed because no essential difference was noticed between these methods The mixture was always prepared immediately before use and it was used only once The staining time varied from 5 to 15 minutes

Diazo Coupling Reaction — Only stable diazotates were used The sections were stained in 0 1 M veronal acetate buffer at pH 9.2 The buffer contained either 0 1 % 5-nitroanisidine (Fast Red Salt B I C.I Ltd) or 0 1 % *p*-nitroaniline (Fast Red Salt GG I C I Ltd) The dye solutions were prepared immediately before use according to Pearse (1960) and no essential difference was observed between the used salts in demonstrating EC. The staining procedure lasted 2—10 minutes

Indophenol Reaction — In principle the technique given by Pearse (1960) was used A 0 1 solution of Gibbs reagent 2,6-dichloro-quinonechloroimide (E Merck AG) was prepared in 0 1 M veronal acetate buffer at pH 9 2. The mixture was warmed five times to 70°C and shaken vigorously The staining was carried out at room temperature for 5 to 10 hours

F o r m a l d e h y d e i n d u c e d F l u o r e s c e n c e — In principle the techniques given by Falck and Torp (1961) and Eranko (1964) were followed Small duodenal pieces were frozen with the aid of metal disc forceps precooled in liquid nitrogen (Eranko 1954) or dropped directly into liquid nitrogen They were dried in vacuo at —35 C to —45 C for 1—4 days After drying the pieces were treated with formaldehyde vapour from paraformaldehyde at +80 C for 1—2 hours in a closed glass vessel The tissues were then embedded in paraffin wax directly under vacuum or after immersion in xylene The sections were cut at 5 micra and were deparaffinized with xylene on slides for fluorescence studies Some sections were attached on slides with a thin layer of fresh egg white and were then dried for the deparaffinization with xylene The fluorescence was registered by microphotography the various staining reactions were carried out and the sections were then rephotographed

For fluorescence studie Wild s monocular microscope was used The light source was a high pressure mercury lamp HBO 200 (Osram) The light was filtered through Schott BG12 and BG23 filters and two Schott K2 heat ab orbing filters The stop filters in the tube were Leitz Euphos and Schott OG1 Sometimes a dark field condenser was used The photographs were taken on Perutz 27 film Exposure times varied between 10 and 120 seconds All optical components were of glass

A u t o f l u o r e s c e n c e R e a c t i o n — The specimens were removed frozen and dried in the same way as in the fluorescence reaction but they were not exposed to paraformaldehyde

QUANTITATIVE METHODS

N u m b e r o f E C — Freeze-dried specimens treated with paraformaldehyde vapour were used for the determination of the EC count Small intestinal pieces 1—2 mm in diameter and 3—4 mm in length were taken from the duodenum of the sheep cow pig horse and rabbit These specimens were cut in the longitudinal direction of the intestine opposite to the mesenterium The specimens taken from the guinea pig mouse and rat were about 3 mm in length and included the whole transverse section surface of the duodenum From each specimen 6—8 paraffin sections were cut at 5 micra In order to avoid inaccuracies due to EC accumulations a distance of 100—200 micra was always left between the different sections All counts were made by the present writer using a magnification of 200—600 × The EC in 5—8 sections were counted and the mean number of the EC per section was defined for each specimen

Other sections immediately adjacent to the counted sections were cut at 10 micra and stained with haematoxylin-eosin (see Romeis 1948) The magnified images (34 or 52 ×) were projected and drawn on paper All drawnings were measured with a planimeter (Eranko 1955b) The mean volume (mm^3) of each specimen was defined With the aid of these mean numbers and mean volumes the EC count per 1 mm^3 of duodenum was determined for each specimen The values obtained were corrected according to the formula of Floderus (Eranko 1955b) The diameter of the fluorescent FC was determined from microphotographs (887×) for each species and each age group The mean diameter defined from 60—104 EC in each case was the mean proportional of the product of the long axis of the EC and their largest breadth perpendicular to it The mean vertical height of the smallest fluorescent structures that could be identified as EC was determined from 20 fluorescent bodies in microphotographs

It was found that there were no EC in the muscularis interna and externa of the sheep cow pig and horse Therefore tissue pieces containing only submucosa and mucosa were taken from these animals to facilitate successful freeze-drying For each animal the relative proportion of the united muscular and the submucous mucous layers was determined in formalin fixed sections

stained with haematoxylin eosin This proportion was taken into consideration when determining the EC count in 1 mm^2 of the duodenum

Estimation of 5 Hydroxytryptamine — The spectrophotofluorometric method of Udenfriend *et al* (1955) as modified by Weissbach (1961) was used for the determination of the 5 HT content in the duodenal pieces The specimens were homogenized in 0 1 N HCl From salt saturated homogenate 5 HT was extracted into butanol and transferred with heptane into 0 1 N HCl Concentrated HCl was added and the measurement was performed The activation peak was at 296 nm and the fluorescence peak at 550 nm 5 Hydroxytryptamine creatinine sulphate (Serotonin-creatinin sulfat Monohydrat Fluka AG) was used as a standard

STATISTICAL ANALYSIS

Student s t test was mainly used in the statistical analysis and in a special case an analysis of variance was also performed (Franko 1955b)

RESULTS

I HISTOCHEMICAL STUDIES

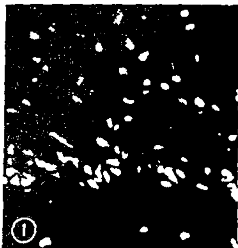
FORMALDEHYDE—INDUCED FLUORESCENCE

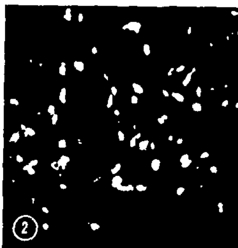
General Observations — In all the species investigated EC showed a strong formaldehyde induced fluorescence No essential differences in the fluorescence intensity were observed between the different duodenal areas or the various species The shape of the EC varied from round to triangular in the basal parts of the mucosa in the villi they were mainly columnar Only in the rabbit the shape varied considerably in the villi where many strikingly narrow and long EC were seen The EC were fairly similar in size in the different species In the guinea pig they were however often a little larger and in the mouse rat and rabbit often smaller than in the other animals

In all species the cytoplasm of the EC was filled with coarse strongly fluorescent granules These granules were most numerous in the basal part of the EC where also the fluorescence was most intense The granules were more easily distinguished in the other parts of the EC because of the weaker fluorescence No essential difference in the size of the granules and in their abundance was noticed between various species in ultraviolet microscopy In all the animals except the horse there were always some EC in which a narrow fluorescent strip of cytoplasm extended to the intestinal lumen Such strips were very numerous in the sheep The nuclei of the EC showed no fluorescence and they were seen as blackish spots in most EC The nuclei were generally in the basal part of the cells

The EC fluorescence was very stable Even when the fluorescence of nerve fibres was diffuse indicating diffusion of catecholamines the EC remained sharply delineated and strongly fluorescent If sections were immersed in water without deparaffinization the fluorescence of most EC did not disappear On the covered object glass without mounting medium the distinct EC fluorescence lasted 6—12 months at room temperature and humidity

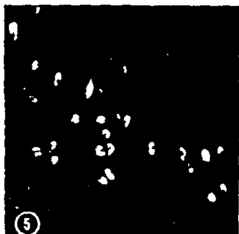
Sheep — The fluorescence microscope field was dominated by a thin and dense band of EC (Fig 1) Most of them were situated in the crypts of Lieberkuhn In each crypt there were in general 5 20 EC, most of them side by side Sometimes aggregations of 100—200 EC were seen Only a few EC were found in the villi and in the submucosa The fluorescence colour was a bright yellow with a faint nuance to brown and it did not weaken after a longer exposure to ultraviolet light


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C o w — All sections of the cow showed several fluorescent EC although the microscope field was dominated by green fluorescent dopamine cells (Fig 2) In each crypt of Lieberkuhn there were 4—12 EC and dense cell accumulations were sometimes seen in this part of the mucosa Elsewhere in the mucosa only some isolated EC were observed and none of them in the submucosa The EC showed a yellow fluorescence with a brownish nuance The fluorescence intensity remained strong even after a long exposure to ultraviolet light

P i g — The EC of the pig were seen as strongly fluorescent clumps in the ultraviolet microscope In every Lieberkuhn s crypt there were 2—10 EC and *sometimes aggregations of 20—30 cells were seen (Fig 3) In the villi and the* submucosa only few EC were observed The colour of the fluorescence in the EC granules was a bright yellow and its intensity remained unchanged after 1 hour s exposure to UV light

H o r s e — Only a few fluorescent EC were seen in the microscope field (Fig 4) They were scattered quite evenly in the basal part of the mucosa Rarely there were two EC side by side and no aggregations were seen The thick clumpish villi contained very few EC and none of them were found in the submucosa After a longer UV illumination the yellow fluorescence of the EC faded to some degree

G u i n e a P i g — The incidence of fluorescent EC was largest in the basal part of the mucosa (Fig 5) In the crypts of Lieberkuhn there were 1—5 EC and seldom larger cell aggregations were seen Isolated EC were noticed also in the submucosa and among the villous epithelial cells where their distribution was quite even The fluorescent EC exhibited a yellow colour with a greenish nuance Some decrease in the fluorescence intensity was observed after 1 hour s UV illumination

M o u s e — Only few EC were seen in the microscope field (Fig 6) Most of them were distributed quite evenly in the basal part of the mucosa and no cell aggregations were seen Most crypts of Lieberkuhn contained no EC The fluorescence was bright yellow in colour After quite a short illumination its intensity weakened more clearly than that in the other species examined

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Formaldehyde induced fluorescence in the duodenum of mammals The basal parts of the duodenum are seen in the lower areas of the figures

Fig 1 Sheep Note the great density of EC in the basal part of the mucosa Most of the fluorescent cells in the superficial part of the mucosa are dopamine cells In the submucosa there are some strongly fluorescent dopamine cells × 160

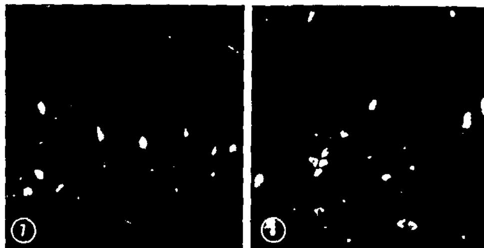
Fig 2 Cow Small EC aggreg tions are se n in the basal part of the mucosa Most of the strongly fluorescent cells in the superficial part of the mucosa are polymorphic dopamine cells × 160

Fig 3 Pig The strongly fluorescent EC are diffusely distributed in the basal part of the mucosa × 160

Fig 4 Horse Most of the few strongly fluorescent EC are located in the basal part of the mucosa × 160

Fig 5 Guinea pig Small strongly fluorescent EC patterns are seen in the basal part of the mucosa Most EC have non fluorescent nuclei × 160

Fig 6 Mouse A few strongly fluorescent EC are seen in the basal and villous parts of the mucosa. Some strongly fluorescent small mast cells are present in the proximal part of the villi × 225



Formaldehyde induced fluorescence in the duodenum of mammals × 160
Fig. 7 Rat In the basal part of the mucosa only some strongly fluorescent EC are seen
Fig. 8 Rabbit In the basal part of the mucosa there are small strongly fluorescent EC aggregations Some degree of polymorphism of EC is seen

R a t — Also in the rat no EC accumulations were observed (Fig 7) Most EC were situated in the crypts of Lieberkuhn One crypt seldom contained two cells Some individual EC were observed among the epithelial cells of the villi The colour of the fluorescence was a bright yellow The intensity weakened quite quickly under ultraviolet light

R a b b i t — As in the other animals the largest EC patterns were in the basal part of the mucosa (Fig 8) However more numerous villous EC were seen in the rabbit than in the other animals studied The distribution of the EC in the mucosa was quite even No larger cell aggregations were seen In the thick submucosa there were some separate EC The bright yellow fluorescence colour faded gradually under ultraviolet action

O t h e r F l u o r e s c e n t S t r u c t u r e s — Few fluorescent mast cells were often seen in the duodenum of the pig horse guinea pig mouse rat and rabbit Their yellow fluorescence was quite similar to that of the EC In the pig horse and rabbit these fluorescent mast cells were more often present in the submucosa In the guinea pig mouse and rat they were most numerous in the central areas of the villi The mast cells were generally located between glandular elements around the blood vessels and were smaller and more irregular in shape than the EC Therefore in spite of the similar yellow fluorescence these two cell types could be easily distinguished from each other

In the duodenum of the sheep and cow there were numerous fluorescent dopamine cells with a greenish granular cytoplasm (Figs 9—12) They were distributed all over the mucosa and submucosa and some of them were also in the myenteric layers and subserosa particularly around the blood vessels In the cow they were especially numerous in the central part of the villi The ratio of the EC and dopamine cells in the duodenum of the sheep was 1 2 and of the





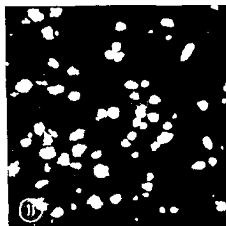




Fig 9 Formaldehyde-induced fluorescence in the cow duodenum Strongly fluorescent EC and dopamine cells are seen in one crypt of Lieberkuhn × 320
Fig 10 The same section after the argyrophil reaction Only the EC are specifically stained by the argyrophil reaction × 320
Fig 11 Formaldehyde induced fluorescence in the cow duodenum Numerous strongly fluorescent EC and dopamine cells are seen. × 160
Fig 12 The same section after the argentaffin reaction Only the EC are specifically stained by the argentaffin reaction × 160

cow 1 2—4 (five specimens were counted in each case) Because of the different fluorescence colours the EC and the dopamine cells were easily distinguished from each other even in a low magnification (100 ×)

A strong greenish fluorescence was seen in the submucous and myenteric nerve plexus of the duodenum Delicate greenish fluorescent nerve fibres were often present in the mucosa and submucosa in the myenteric layers and in the subserosa particularly around the smaller arteries and arterioles This nerve fluorescence was an indication of a successful freeze-drying procedure No connection was discerned between the fluorescent EC and nerve fibres

In all species there were granular brownish yellow fluorescent cells located in the lamina propria of the intestinal villi and Payer's patches Because of their location and different fluorescence properties these cells clearly differed from the EC Their fluorescence was stable and it was also seen in the specimens not exposed to formaldehyde.

The duodenal mucous surface often contained a thin fluorescent layer of mucus varying in colour from yellow to red In the intestinal lumen there was often fluorescent material of various colours Both these types of fluorescence were mostly due to autofluorescence

The background of the sections showed generally a very weak dirty green fluorescence

A yellow fluorescence similar to that of the EC and the mast cells was not observed anywhere else in the duodenum

STAINING REACTIONS

General Observations — In each species the specifically coloured EC in the staining reactions were always situated in the same areas of the mucosa and submucosa as in the fluorescence reaction The largest patterns of stained EC were seen in the basal part of the mucosa especially in the crypts of Lieberkuhn Separate EC were observed in the villi and in the submucosa In the muscular and subserous layers there were no positive EC. All the EC in each species stained strongly positive in the argyrophil reaction In the other staining reactions the cytoplasmic colour intensity of EC varied considerably but no general difference was noticed between the villous and basal EC in the staining intensity All the reactions were located in coarse granules in the cytoplasm of the EC and the granules were most numerous in the basal part of the cells The shape and size of the stained EC exactly corresponded to those of the fluorescent EC On the other hand fluorescent mast cells failed to exhibit positive silver reactions and also in the dopamine cells these reactions were very weak in comparison to those of EC (Figs 9—12) In the other staining reactions these cell types stained as the background

Argyrophil Reaction — In all species the EC were stained a strongly deep blue or black and no essential difference in the colouring of the EC was observed between the species investigated The EC were easily distinguishable from the other intestinal cells (Figs 13—19 and 56) Especially in the sheep pig and rabbit there were often seen narrow and strongly coloured cytoplasm strips extending to the intestinal lumen Such strips were not observed in the horse and the mouse

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Argyrophil reaction in the duodenum of mammals × 160

Fig 13 Sheep Numerous strongly stained EC are seen in the crypts of Lieberkühn

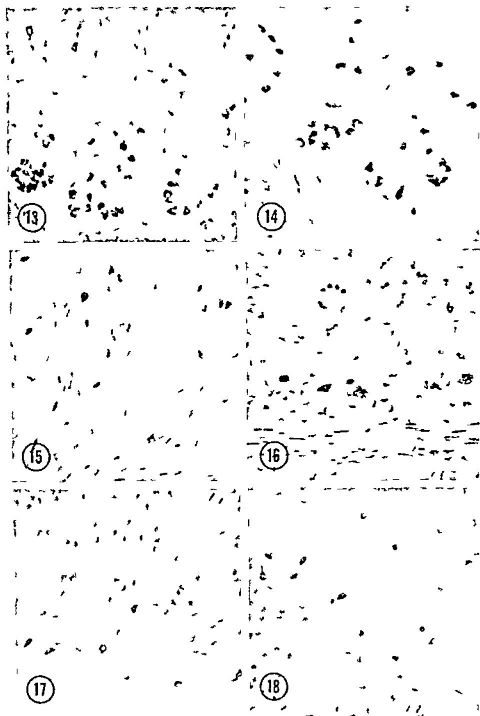
Fig 14 Cow Numerous strongly stained EC are seen in the crypts of Lieberkühn.

Fig 15 Pig Some isolated strongly stained EC are seen in the crypts of Lieberkuhn

Fig 16 Horse A few strongly stained EC are seen in the crypts of Lieberkuhn

Fig 17 Mouse Some strongly stained EC are seen in the crypts of Lieberkuhn The section taken near the pylorus

Fig 18 Rat Some strongly stained EC are seen in the crypts of Lieberkuhn


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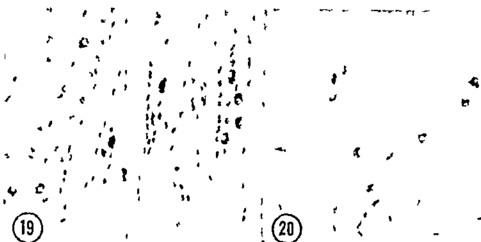


Fig. 19. Argyrophil reaction in the rabbit duodenum. Some strongly stained EC are seen in the crypts of Lieberkühn. × 160.
Fig. 20. Argentaffin reaction in the rabbit duodenum. Some of the EC in the crypts of Lieberkühn are more strongly stained. × 160.

The argyrophil reaction stained the cytoplasm of all the other cells very weakly although the staining time was long. On the other hand, the nuclei of the other cells became moderately blue, sometimes reddish. If the staining time was too long, the whole section turned blackish because of the great density of nuclei.

The nerve plexus stained moderately blue in each species and was easily distinguished in the muscular layers and submucosa. In the mucosa delicate nerve fibres were seen, but they were in no connection with EC. Unspecific silver precipitates accumulated in almost every section. These stained clumps were easily distinguished from the stained tissues.

A r g e n t a f f i n R e a c t i o n. — The intensity of the argentaffin reaction in EC was strong in the sheep, cow, pig, horse and guinea pig, moderate in the rat and rabbit, and weak in the mouse (Figs. 20—26 and 66). The colour varied from black to light brown. In the pig and rabbit, narrow cytoplasmic strips extended to the intestinal lumen. The nuclei were only slightly stained.

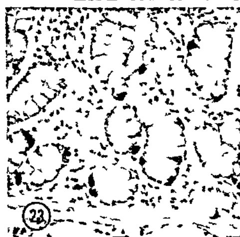
The neighbouring tissue was in general weakly stained. The colour varied in different nuances of brown. If the staining time was long, the whole section turned black. The nuclei of the other cells were stained a slightly deeper brown than

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Argentaffin reaction in the duodenum of mammals. × 160.
Fig. 21. Sheep. Numerous strongly stained EC are seen in the crypts of Lieberkühn.
Fig. 22. Cow. Numerous strongly stained EC are seen in the crypts of Lieberkühn.
Fig. 23. Pig. Some strongly stained EC are seen in the crypts of Lieberkühn.
Fig. 24. Horse. A few strongly stained EC are seen in the crypts of Lieberkühn.
Fig. 25. Mouse. Most of the EC are weakly stained. The section was taken close to the pylorus.
Fig. 26. Rat. Some of the EC are more strongly stained. The section was taken near the pylorus.


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Ferric Ferricyanide Reaction. — The staining intensity of the EC varied considerably between the different species. It was very strong in the pig and guinea pig, moderate in the cow and rabbit, and weak in the sheep, horse and rat (Figs. 27—29 and 70). In the mouse only a few EC were positive. In the duodenum of the mouse and rat the stained EC were most numerous near the pylorus, where also the reaction was more intense than in the other parts of the duodenum. In all species the colour of the coarse EC granules varied from deep blue to light green and the narrow apical parts of the EC as well as the nuclei were almost colourless.

All reactive EC were easily discerned among the other cells. The background was in general light green and its colour depended on the staining time. After a longer reaction time the sections became a deep blue. The nuclei of the other cells exhibited a more intense reaction than their cytoplasm.

Diazo Coupling Reaction. — This reaction was strong in the pig and guinea pig, weak in the sheep, cow and rabbit, but just visible in the horse, mouse and rat (Figs. 30—32 and 74). In the mouse and rat reactive EC were mostly seen near the pylorus, where also the reaction intensity was a little stronger. The colour of the EC varied from brownish red to light yellow. The apical parts of the EC were almost colourless.

In general the background was stained light yellow, but after a longer staining it turned a deep brown. The strongly coloured EC were easily distinguishable. No essential colour difference was seen between the cytoplasm and the nuclei in the other cells. In the blood vessels and in the central part of the villi there were often small strongly stained cells, which were easily distinguished from the EC.

Indophenol Reaction. — The indophenol reaction was negative in the EC of the horse, mouse and rat (Fig. 35). Also in the cow and rabbit the reactive EC were only just visible. In the sheep (Fig. 33) the reaction intensity was moderate, whereas in the pig (Fig. 34) and guinea pig (Fig. 78) it was strong. The colour of the reaction was grey or blackish. The apical parts of the EC cytoplasm were almost colourless in each species. The nuclei of the EC stained very weakly.

The background of the sections remained almost colourless and the stained EC were easily discerned as dark points in the sections. After several hours staining also the background turned a dirty grey and especially some cells in the blood vessels were stained blackish. The nucleus and cytoplasm of the other cells were similarly intensively stained.

AUTOFLUORESCENCE

The EC showed no autofluorescence in the species investigated.

The results obtained with the different reactions are summarized in Table 1. The mean fluorescence and staining intensity of the EC in the various species was labelled as follows: negative —, just visible +, weak + +, moderate + + +, strong + + + +. All the reactions were strongly positive in the EC of the guinea pig. Therefore the average intensity in this animal was recorded in each reaction as + + + +. The intensity of the different reactions in the EC of the other species was compared with that of the guinea pig.

TABLE 1 *Histochemical reactions in the EC of mammals*

For explanations see the text. The figures in brackets indicate the number of animals studied

Reactions	Sheep (8)	Cow (10)	Pig (8)	Horse (6)	Guinea Pig (9)	Mouse (10)	Rat (8)	Rabbit (7)
Formaldehyde induced fluorescence	++++ OOOO	++++ OOOO	++++ OOOO	++++ OOOO	++++ OOOO	++++ OOOO	++++ OOOO	++++ OOOO
Argyrophil	++++ OOOO	++++ OOOO	++++ OOOO	++++ OOOO	++++ OOOO	++++ OOOO	++++ OOOO	++++ OOOO
Argentaffin	++++ OOOO	++++ OOOO	++++ OOOO	++++ OOO	++++ OOOO	++ OO	+++ OO	+++ OOO
Ferric ferricyanide	++ OOO	+++ OOO	++++ OOOO	++ OO	++++ OOOO	+ O	++ O	+++ OO
Diazo coupling	++ OOO	++ OOO	++++ OOOO	+ O	++++ OOOO	+ O	+ O	++ O
Indophenol	++ OO	+ O	++++ OOOO	— —	++++ OOOO	— —	— —	+ O
Autofluorescence	— —	— —	— —	— —	— —	— —	— —	— —

The n u m b e r of fluorescent EC was indicated by the sign o o o o In the different staining reactions the number of positive EC was compared with that of the fluorescent EC and the result was designated as follows no positive EC — few positive EC o some positive EC o o numerous positive EC o o o as many as fluorescent EC o o o o

Serial sections taken from each specimen were used for the calculation of these mean amounts of EC and their average reaction intensity (magnification 200 or 400 ×) These calculations are summarized in Table 1

It is quite clear that there is a correlation between the number of EC shown by the various methods and the intensity of these reactions in the EC *i e* that methods giving a weak reaction also make fewer EC visible

DISCUSSION

All the species investigated showed numerous EC in the duodenum This observation is in agreement with many earlier studies (Moller 1899 Tang 1922 Hamperl 1925 Kull 1925 Cordier 1926 Toro 1929 Eros 1930 Tehver 1930 Clara 1933a Jacobson 1939)

Silver Reactions

In each species all the EC stained strongly in the argyrophil reaction On the other hand in the argentaffin reaction the staining intensity of the EC varied in different species In the sheep cow horse and guinea pig the EC exhibited an intense argentaffin reaction whereas the reaction intensity was weaker in the other animals Besides in the horse mouse rat and rabbit the EC in the argentaffin reaction were less numerous than those in the argyrophil reaction This kind of distinct difference in the number of the argyrophil and argentaffin EC has been earlier observed by Dawson (1945 1948) in the stomach of several laboratory animals by Hellweg (1952) Hamperl (1952) Hardmeier and Hedinger (1965) and Singh (1965a) in the human gastrointestinal tract as well as by Eder *et al* (1959) and Prellwitz (1959) in the duodenum of the guinea pig According to all these investigations the argyrophil EC were more numerous than the argentaffin EC

In the present study it was however observed that in the sheep cow pig and guinea pig the argentaffin EC were as numerous as the argyrophil EC This observation is obviously due to the fact that the tissue pieces were taken close to the pylorus for it was stated by Tehver (1930) Clara (1933a) and Singh (1965a) that in the proximal part of the duodenum the argentaffin EC were more numerous and more easily demonstrable than in the other duodenal parts Obviously the EC near the pylorus contain a lower turnover rate of 5 HT and much more substances which have argentaffin properties than the EC in the other duodenal areas

The great difference between the argyrophil and argentaffin reactions in demonstrating the EC must be caused principally by dissimilar properties in the EC granules or the argyrophil reaction is a much more sensitive one The argyrophil EC may be the precursors of the argentaffin EC as postulated by Erspamer (1937) or they may be in another physiological state Because of the lack of chemical specificity of the argyrophil reaction the substance causing this reaction

in the EC is entirely obscure but it is of interest that it remains in the entero chromaffin granules even after alcohol fixation (Eder *et al* 1962) when the other staining reactions are negative However the argyrophil reaction is more reliable in demonstrating the EC than the more specific argentaffin reaction

Other Staining Reactions

Great differences were observed between the various reactions with respect to the staining ability of the EC The silver reactions were more effective for demonstrating the EC than either the ferric ferricyanide or the diazo coupling reaction The indophenol reaction was distinctly less effective than all the other reactions However all the reactions were strongly positive in the EC of the pig and guinea pig In the other species the ferric ferricyanide and diazo coupling reactions stained the EC less effectively and the indophenol reaction was entirely negative in the EC of the horse mouse and rat

The results obtained by earlier investigators are equally variable The argent affin ferric ferricyanide and diazo coupling reactions were demonstrated to be equally effective in the staining of the EC in the human appendix (Laskey and Greco 1948) in the duodenum of the guinea pig (Prellwitz 1959) and in the hu man large bowel (Hardmeier and Hedinger 1965) On the other hand Eder *et al* (1959) demonstrated that in the duodenum of the guinea pig the argentaffin reac tion stained about twice as many EC as the ferric ferricyanide diazo coupling and indophenol reactions Only some of the EC that were positive in the ferric ferricyanide reaction were also diazo positive according to Lillie *et al* (1961) However the results obtained in the present study showed that the reaction intensity and the EC count are in a clear correlation to each other in mammalian species

It has been in general assumed that all the staining reactions with the exception of the argyrophil reaction are caused by the same substance in the EC *i e* a con densation product of 5 HT and formaldehyde (Barter and Pearse 1955 Pearse 1960) The intensities of the staining reactions show a direct correlation with the 5 HT concentration as was demonstrated by Benditt and Wong (1957) in gelatine models It seems therefore that the 5 HT content of the EC determines the reaction intensity in great degree The influence of the precursors of 5 HT and other chemical substances on the staining properties of the EC is obscure Also the granular structure and the mechanism by which the amines are bound to the granules may be different in various species and cause staining differences Besides the physiological state of the animals the secretory and metabolic activity of the EC the anatomical point of the tissue pieces as well as the staining method used certainly have an influence on the colour intensity of the EC

Fluorescence Reaction

The fluorescence reaction was golden yellow in intestinal EC fixed in formalde hyde solutions (Eros 1932) The sensitivity of the reaction in this method was equivalent to that of the staining reactions (Benditt and Wong 1957)

In the present study the EC exhibited a very strong formaldehyde induced fluorescence in all the species investigated The fluorescence colour was bright

yellow The tendencies towards brown or green in the EC of the sheep cow and guinea pig obviously were due to different concentrations of amines or other substances disturbing the fluorescence

According to Falck and Owman (1965) the yellow fluorescence colour is specific for certain tryptamine derivatives However, subjective estimation of the fluorescence colour is subject to considerable errors and therefore it is better not to depend too much on the colour characteristics Falck and Owman observed that the 5 HT fluorescence disappeared more rapidly than the catecholamine fluorescence In the present study no such essential weakening of the EC fluorescence was observed even after a long exposure to UV light and the speed of fluorescence fading is not a good criterion for 5 HT

In view of the contact between the narrow but always strongly yellow fluorescent cytoplasmic strips and the intestinal lumen it seems likely that the EC also have an exocrine secretory function as has been suggested by Erspamer (1961) Besides the coarse granularity of the EC cytoplasm shows a granular form of storage of the biologically active amines

In the duodenum of different species a yellow fluorescence of this kind was observed only in the EC and mast cells The nerve plexus exhibited only a greenish fluorescence Therefore it is quite possible that most if not all of the intestinal 5 HT is concentrated in these cells *in vivo* With the aid of the fluorescence reaction it is however impossible to determine whether there are other fluorescent tryptamine derivatives in the EC

It has been demonstrated that the mast cells of the mouse and rat contain considerable amounts of 5 HT whereas this amine has been considered to be lacking from the mast cells of other species (Benditt *et al* 1955 Parrat and West 1957 Falck 1962) In the present study mast cells with a strong yellow fluorescence were found in the duodenum of the pig horse guinea pig mouse rat and rabbit The intense yellow fluorescence suggests that tryptamine derivatives obviously 5 HT are a normal component of the mast cells The small 5 HT content of the mast cells was probably overlooked in the earlier studies because the methods used were not adequately sensitive

Falck *et al* (1959) found in the gut of ungulates cells which presumably contained dopamine Coupland and Heath (1961) regarded these cells as typical mast cells In the present study such cells were found in the sheep and cow duodenum The fluorescence of these cells was green and distinctly differed from the yellow fluorescence of the EC and it was probably due to dopamine Because very numerous dopamine cells were seen only in the sheep and cow in contrast to the very few mast cells in all the other species it is likely that their physiological function is different from that of the mast cells It is moreover open to question whether the dopamine cells are true mast cells as claimed by Coupland and Heath (1961) However both cells were not specifically stained in the various staining reactions

II CONSECUTIVE DEMONSTRATION OF FORMALDEHYDE-INDUCED FLUORESCENCE AND STAINING REACTIONS IN ENTEROCHROMAFFIN CELLS

RESULTS

After microphotographs were taken from the fluorescent EC the same sections were stained with the specific reactions and rephotographed

Argyrophil Reaction — In all the eight species all the fluorescent EC exhibited a strongly positive silver impregnation reaction (Figs 36 and 37)

Argentaffin Reaction — In the sheep cow pig and guinea pig (Figs 38 and 39) all the fluorescent EC were also argentaffin In the horse and rabbit most fluorescent EC were positive also in the argentaffin reaction whereas in the mouse and rat a few fluorescent EC were argentaffin

Ferric Ferricyanide Reaction — The comparison was made only in the pig and guinea pig In both animals all the fluorescent EC showed a positive ferric ferricyanide reaction (Figs 40 and 41)

Diazo Coupling Reaction — The correlation was studied only in the pig and guinea pig All the fluorescent EC were stained by the diazo coupling reaction However some of the fluorescent EC were less intensively stained (Figs 42 and 43)

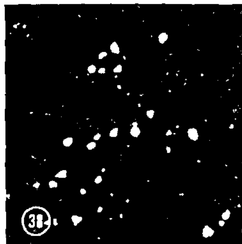
Indophenol Reaction — All the fluorescent EC of the pig and guinea pig duodenum were stained by this reaction (Figs 44 and 45) However the staining of some EC was weak particularly in the pig

DISCUSSION

Whether the same EC are reactive towards all different staining reactions has been hardly examined The earlier observations were made only in the adjacent sections which is a method of questionable value Singh (1963 1964a) used double staining He first stained the EC of the foetal and adult human gastrointestinal tract using either the argentaffin ferric ferricyanide or diazo coupling reaction Thereafter the sections were stained by the argyrophil method Singh demonstrated that all the EC positive in the argentaffin ferric ferricyanide and diazo coupling reactions were also stained in the argyrophil reaction even though the argyrophil EC were more numerous than the positive EC in the other reactions However it is not clear how much the first reaction affected the second one possibly causing an erroneous positive reaction


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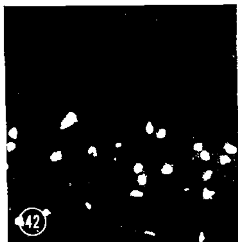

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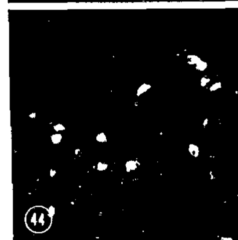

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Consecutive demonstration of formaldehyde induced fluorescence and staining reactions in EC × 160
Fig 42 Formaldehyde induced fluorescence in the guinea pig duodenum
Fig 43 The same section after the diazo coupling reaction All the fluorescent EC are specifically stained also in the diazo coupling reaction
Fig 44 Formaldehyde induced fluorescence in the guinea pig duodenum
Fig 45 The sam section after the indophenol reaction All the fluorescent EC are specifically stained also in the indophenol reaction. Some fluorescent EC are weakly stained

Consecutive demonstration of formaldehyde induced fluorescence and staining reactions in EC × 160
Fig 36 Formaldehyde induced fluorescence in the guinea pig duodenum
Fig 3 The same section after the argyrophil reaction All the fluorescent EC are specifically stained also in the argyrophil reaction
Fig 38 Formaldehyde induc d fluorescence in the guinea pig duodenum
Fig 39 The same section after the argentaffin reaction All the fluorescent EC are specifically stained also in the argentaffin reaction
Fig 40 Formald hyd induced fluorescence in the guinea pig duodenum
Fig 41 The same section after the ferric ferricyanide reaction All the fluorescent EC are specifically stained also in the ferric ferricyanide reaction

The fluorescence reaction has not previously been correlated with the staining reactions although it offers unique possibilities for such a comparison since the second reaction can be applied to an unstained section

In the present study it was undeniably demonstrated that the EC in all the mammals investigated were composed of morphologically similar fluorescent cells It was further noted that all the fluorescent EC were positive also in the argyrophil reaction In the pig and guinea pig all the other staining reactions also were positive in the same EC as the fluorescence reaction as was already indicated by cell count calculations in serial paraffin sections Only some of the fluorescent EC exhibited a positive argentaffin reaction in the horse mouse rat and rabbit whereas in the sheep and cow they all were also argentaffin The stainings performed after the fluorescence reaction excluded the possibility that there were duodenal EC which were positive only in the argentaffin reaction without argyrophil properties as postulated by Hellweg (1952) and Hamperl (1952) On the other hand, in all the species investigated there were no stained EC without a yellow fluorescence

The yellow colour of the fluorescence suggests that the enterochromaffin system at least in mammals is composed of cells all containing similar chemical substances The stages of the pre enterochromaffin non argyrophil and empty EC suggested by Erspamer (1937) were not demonstrable in the present study with the highly sensitive fluorescence reaction which observation abolishes Erspamer s theory at least in adult mammals It was also impossible to find any intermediate cell forms between the EC and intestinal epithelial cells from which the EC are obviously derived (Cordier 1926 Friedmann 1934) Therefore it is probable that in the adult mammals the enterochromaffin system is composed of one cell type only the principal properties of which are the yellow formaldehyde induced fluorescence and the strong argyrophilia

III ENTEROCHROMAFFIN CELLS AND 5 HYDROXYTRYPTAMINE IN THE DUODENUM

CORRELATION BETWEEN THE NUMBER OF ENTEROCHROMAFFIN CELLS AND THE 5 HYDROXYTRYPTAMINE CONCENTRATION

Material

A total number of 109 animals were studied. The EC were counted and the 5-HT content was determined in the duodenal specimens of 23 sheep 14 cows 17 pigs 12 horses 10 guinea pigs 16 mice 11 rats and 6 rabbits If the freeze drying procedure was not successful, the specimens were rejected

Results

The diameter of the fluorescent EC was 10 7 μ in the sheep 10 3 in the cow 11 1 in the pig 10 8 in the horse 11 7 in the guinea pig 9 5 in the mouse 9 7 in the rat and 9 9 in the rabbit The smallest vertical height of the fluorescent structures which could be identified as EC was 3 μ

Enterochromaffin Cells — The largest mean value of the EC (7 040 EC/mm^3 tissue) was observed in the duodenum of the guinea pig Also in the sheep and rabbit the EC were remarkably numerous Their mean values (6 260/mm^3 and 4 940/mm^3) however differed significantly ($P<0\ 05$ and $P<0\ 001$ respectively) from the mean value of the guinea pig and from each other ($P<0\ 01$) The mean EC counts of the rat (3 340/mm^3) cow (2 970/mm^3) pig (2 450/mm) and mouse (2 170/mm^3) were of the same size class The EC count of the horse (850/mm^3) differed considerably ($P<0\ 001$) from all the other animals The standard deviations varied between 9 8 % and 25 4 % (Fig 46)

5 Hydroxytryptamine — The highest 5 HT concentrations were found in the sheep (the mean value 24 9 μg/g tissue) and the guinea pig (19 2 μg) The 5 HT contents were also considerably high and almost equally large in the cow (13 9 μg) the pig (13 1 μg) and the rabbit (11 0 μg) All the other animals showed distinctly smaller 5 HT concentrations However the mouse (6 3 μg) differed significantly ($P<0\ 001$) from the horse (3 2 μg) and the rat (3 0 μg) The standard deviations varied from 11 9 % to 49 4 % (Fig 46)

Correlation between the Enterochromaffin Cells and 5 Hydroxytryptamine — The relationship is represented in Fig 47 with a corresponding regression line defined from the mean values for each species The regression equation is $y = 1\ 25 + 0\ 0028\ x$ with a correlation coeffi

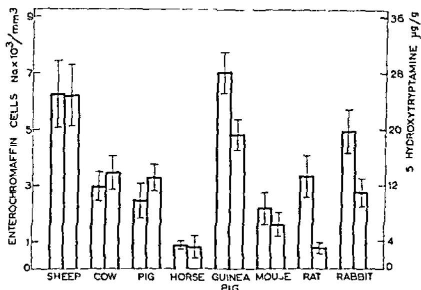


Fig 46 The number of EC and the 5-HT concentration in the duodenum of mammals Ordinate left EC No $\times$ 10^3/mm duodenal tissue Ordinate right 5 HT concentration μg/g duodenal tissue Abscissa animal species EC hatched 5-HT unhatched Means and standard deviations

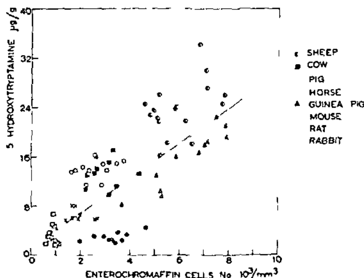


Fig 47 Regression line demonstrating the relationship between the EC number and the 5-HT concentration in the duodenum of mammals Ordinate left 5-HT concentration μg/g duodenal tissue Abscissa EC No $\times$ 10^3/mm^3 duodenal tissue $y = 1.25 + 0.0028\,x$ correlation coefficient $r = 0.79$ which is highly significant ($P < 0.001$ $n = 109$)

cient r = 0 79 which is highly significant ($P<0\,001$ n =109) The individual points of the sheep cow and pig are above the regression line whereas those of the rat and rabbit are all below it Only the points of the horse guinea pig and mouse are distinctly on both sides of the regression line

MEAN 5 HYDROXYTRYPTAMINE CONTENT OF THE ENTERO CHROMAFFIN CELLS

It was assumed that all the 5 HT in the duodenum located in the EC The calculation of the mean 5 HT content in one EC was based on the EC counts 5 HT concentrations and specific gravities of the duodenum and was made by dividing the 5 HT content by the EC number in the same tissue volume

The specific gravity of the duodenal tissue was 1 058 in the sheep 1 056 in the cow guinea pig and rabbit and 1 055 in the pig horse mouse and rat The specific gravity was determined in sucrose solutions The table published by Geigy (1960) was used

The EC of the pig cow sheep and horse showed the largest mean 5 HT content in one EC (Fig 48) The mean values in these species varied in the range 5 9—4 1 × 10^{-6} µg 5 HT In the mouse guinea pig and rabbit the mean values were smaller and varied from 3 3 to 2 4 × 10^{-6} µg In comparison to all the other animals the 5 HT concentration was very low in the EC of the rat (1 2 × 10^{-6} µg) and differed very *significantly ($P<0\,001$) from them The standard deviations* varied between 12 3 % and 47 1 % and were considerably high in the horse and mouse

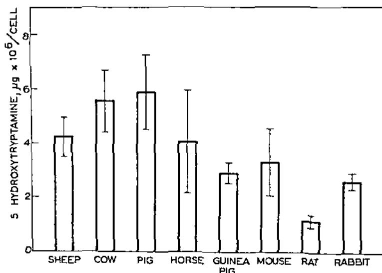


Fig 48 The mean content of 5 HT in the EC of mammals Ordinate 5-HT µg × 10^{-6}/cell Abscissa animal species Means and standard deviations

DISTRIBUTION OF THE ENTEROCHROMAFFIN CELLS AND 5-HYDROXYTRYPTAMINE IN THE DIFFERENT DUODENAL LAYERS

Material

Thirteen cows were used in the experiment because the duodenal wall was easily separated into two layers only in this animal. One of these layers consisted of the mucosa and submucosa and the other of the muscularis interna, muscularis externa and serosa layers. The ratio between the volumes of the separated layers was 1.00 : 1.01. With the aid of the usual haematoxylin-eosin staining it was confirmed that the separated layers comprised only the components stated.

Results

The EC were observed neither in the muscularis externa or interna nor in the serosa or subserosa. The mean 5-HT content in these layers together was 1.2 μg/g tissue. On the other hand, in the united mucous-submucous layer the mean EC count was 5 900 EC/mm³ tissue and correspondingly also the mean 5-HT concentration in this layer was considerably high (22.9 μg 5-HT/g tissue). The values for the mucous-submucous layer were about twice as high as for the whole duodenal wall (Fig. 49).

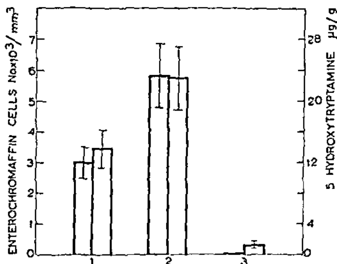


Fig. 49. The number of EC and the 5-HT concentration in the different layers of the cow duodenum. Ordinate left: EC No. × 10³/mm³ duodenal tissue. Ordinate right: 5-HT concentration μg/g duodenal tissue. Group 1: the whole duodenal wall; group 2: the united mucous and submucous layers; group 3: the united internal muscular, external muscular and serous layers. EC hatched, 5-HT unhatched. Means and standard deviations.

ENTEROCHROMAFFIN CELLS AND 5-HYDROXYTRYPTAMINE IN THE DEVELOPING DUODENUM

Material

A total number of 54 rats and 9 guinea pigs were studied. The rats were 1—2 hours (8 rats), 4 days (9), 8 days (5), 12 days (5), 16 days (11), 24 days (5), 32 days (5), and 56 days (6) of age

The nine guinea pigs were studied immediately after birth The EC counts and the 5-HT con centrations in the duodenum were determined separately for each age group

Results

Rat — Strongly fluorescent yellow EC were seen at all ages of the rat No difference in the fluorescence intensity of the EC was observed between the various ages The size and shape of the EC in the newborn rat were quite similar to those in the older animals However it was noticed that the villous EC were proportionally more numerous in the younger than in the older animals Few yellow fluorescent mast cells were seen especially in the lamina propria of the villi This kind of yellow fluorescence was not observed in the other tissue components

The diameter of the fluorescent EC determined for the Floderus correction was 9 9 μ in the newborn rat 10 0 in the 4 day old 9 8 in the 8 day old 9 6 in the 12 day old 9 7 in the 16 day old 10 0 in the 24-day old and in the 32 day old and 9 7 in the 56 day old rat

The EC count was significantly higher in the younger than in the older rats (Fig 50) Immediately after birth and at the age of 4 days great individual variations were observed in the number of EC The mean values for these ages were 5 260 (EC/mm³ tissue) and 6 570/mm³ the standard deviations 27 2 % and 16 9 % respectively Thereafter a clear decrease was observed A significant increase in the EC count was observed at the age of 16 days ($P<0\ 001$) A continual decrease in the EC count was noticed at the age of 24 and 32 days when the adult level was reached

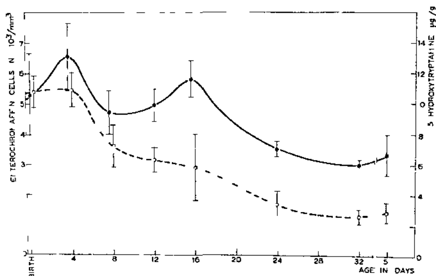


Fig 50 The number of EC and the 5 HT concentration in the developing rat duodenum Ordinate left EC No × 10³/mm duodenal tissue Ordinate right 5-HT concentration μg/g duodenal tissue Abscissa age in days EC marked — — — — — 5 HT marked —o—o— Each point is a mean value ± standard deviation

A similar decrease was observed in the 5 HT concentration It was at its highest in the newborn and 4-day old rats the mean values being 10 8 and 10 9 (μg 5 HT/g tissue) Thereafter a continual decrease was observed and the adult level was reached between the ages of 24 and 56 days (Fig 50)

Guinea Pig — In the duodenum of the guinea pig numerous strongly fluorescent yellow EC were observed immediately after birth They were mainly situated in the basal part of the mucosa Some separate EC were also found in the villi No difference in the size and shape or the fluorescence intensity of the EC was noticed between the newborn and adult guinea pigs Except for few fluorescent mast cells no other yellow fluorescent components were seen

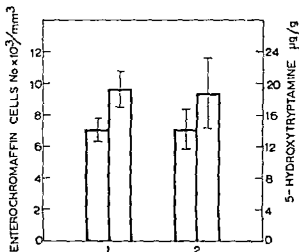


Fig 51 The number of EC and the 5 HT concentration in the duodenum of the guinea pig Ordinate left EC No × 10³/mm³ duodenal tissue Ordinate right 5 HT concentration μg/g duodenal tissue Group 1 adult guinea pigs group 2 newborn guinea pigs EC hatched 5-HT unhatched Means and standard deviations

No significant difference was noticed in the EC diameter between the newborn and adult guinea pigs the mean values being 11 3 and 11 7 μ respectively

The relationship between the EC count and the 5 HT concentration in the newborn and the adult guinea pig is seen in Fig 51 The mean values of EC (7 090 EC/mm³ tissue) and 5 HT (19 4 μg/g tissue) in the newborn guinea pig were quite similar to those in the adult although the standard deviations were larger in the newborn animals

DISCUSSION

Enterochromaffin Cells

The number of EC has been expressed in terms per section of stated size (Tehver 1930 Hoeschen 1937 Klemm 1937 Vetter 1937 1938 Munch 1939) per visual field of stated size (Benditt and Wong 1957 Sullivan 1960 Funk *et al* 1966a Funk *et al* 1966b) per one or several intestinal crypts (Verity *et al* 1962 Deschner 1965 Funk *et al* 1966a Funk *et al* 1966b), per square millimeter of gut surface per square millimeter of muscularis mucosae or per square milli

meter of basement membrane (Singh 1965b) With the aid of these methods however it is very difficult to make precise quantitative comparisons between the EC counts in different species or different parts of the intestinal tract because there are considerable variations in the intestinal structure and proportionate share of intestinal layers in the different species I have not been able to find any previous study in which the number of EC has been expressed in terms of volume of intestinal tissue

All the EC counts in the present study were expressed per unit volume of the whole intestinal wall which facilitates also the comparison of the EC density and the 5 HT concentration in the duodenum Owing to the thinness of the sections (5 micra) a great number of the counted EC in the sections were cell fragments and the primary results clearly exceeded the true values Therefore it was necessary to correct the results according to the Floderus formula (Eranko 1955b) For the use of this formula the particles must be of spherical form and randomly distributed in the tissue The counts determined in the present study are consequently approximate because the precise determination of the diameter of the EC was difficult in view of the mainly columnar cell shape In general the EC diameter was of the same size class in the different species and the various age groups Only the pig and guinea pig differed clearly from the other animals

The argentaffin reaction used in all calculations in the literature is less suitable for the demonstration of the EC in some animal species and some parts of the alimentary canal as was shown above in the present study Therefore only the formaldehyde induced fluorescence reaction was used for the determination of the EC counts

All the adult animals showed numerous fluorescent EC in their duodenum The highest EC counts were found in the guinea pig (7 040 EC/mm³ duodenal tissue) and in the sheep Considerably lower numbers of EC were observed in the rabbit whereas in the rat cow pig and mouse the EC levels were of the same order of magnitude The small EC density of the horse (850/mm³) differed significantly ($P<0\ 001$) from that of all the other animals These observations lend further support to the investigations made by Tang (1922) Kull (1925) Cordier (1926) and Tehver (1930) According to their studies the EC were most numerous in the duodenum of the pig guinea pig and sheep and moderately numerous in the cow and rabbit while in the horse a few EC were found However according to Kull (1925) and Cordier (1926) the EC were very few in the mouse and rat and it was reported by Hoeschen (1937) and Munch (1939) that they were 5—8 times as numerous in the proximal part of the duodenum of the guinea pig as of the rat The inconsistency of these results is probably due to the inadequacy of the argentaffin method to stain all the EC in the mouse and the rat When the formaldehyde induced fluorescence reaction is used as in the present study the variations are not so great

In the present study all the EC counts of the domestic animals and rabbit were made from tissue pieces taken adjacent to the pylorus whereas in the guinea pig mouse and rat the tissue pieces were cut from the middle of the first third of the duodenum This proximity of the pylorus makes the EC counts considerably high as already shown by Hoeschen (1937) and Munch (1939) using the argentaffin reaction in the guinea pig and rat In the sheep cow pig horse and rabbit the proportion of the muscular and submucous layers in the intestinal

wall is significantly larger than in the other animals These two inane layers compensate for the large species differences in the EC patterns seen solely in the mucosa when defining the EC count for the whole duodenal wall

Schumann (1939) counted the EC by the section technique and found more numerous duodenal EC in the adult than in the newborn guinea pig However this kind of difference was not observed in the present study Besides a considerably higher EC count was noted in the duodenum of the newborn (5 260 EC/mm³ tissue in mean) than in the adult rat (3 340/mm³) During development the EC count decreased continuously and the adult level was reached at the age of 24—32 days A very significant peak in the EC count was observed in the 16 day old rats which obviously indicates alterations at that age in the proportionate amounts of duodenal wall components This kind of difference between newborn and adult animals in the EC counts was also observed by Tehver (1930) in the duodenum of the calf and the foal

5 Hydroxytryptamine

The 5 HT concentrations obtained in the earlier investigations vary considerably according to the animal species the physiological state of the animals the anatomical part of the intestinal tissue and the extraction and assay methods The concentration of 5 HT (in µg/g tissue) in the small intestine has been reported in the sheep in the range 2 00—4 10 in the calf 3 00—3 60 in the pig 0 50—4 00 in the horse 0 32—0 72 in the guinea pig 3 4—14 4 in the mouse 1 0—1 6 in the rat 1 2—6 5 and in the rabbit 3 3—10 7 (Erspamer 1953 1954 1961 Faustini 1955 Weissbach *et al* 1958 West 1958 Bertaccini 1959 Toh 1960 Tissari 1966)

In the present study the values obtained in the duodenum were considerably higher The highest values were observed in the sheep (mean value 24 9 µg 5 HT/g tissue) and the guinea pig In the cow pig rabbit and mouse the mean values were significantly lower and in the horse (3 2 µg) and the rat (3 0 µg) the lowest of all the animals studied Erspamer (1953) and Faustini (1955) also demonstrated that in several animals the proximal parts of the small intestine showed considerably higher concentrations of 5 HT than the distal areas Therefore the high values of the present study are not surprising

In the newborn guinea pig the 5 HT concentration in the duodenum was quite similar to that of the adult guinea pig This supports the observations made by Tissari (1966) It is therefore amazing that the duodenal 5 HT concentration was 160 % higher in the newborn than in the adult rat Such a difference has not been observed earlier On the contrary Bertaccini (1958) reported that the 5 HT content increased during development in the large intestine of the rat It is probable that the decrease of 5 HT observed in the rat duodenum during growth is caused by the commencement of intestinal function (Bulbring and Crema 1959) or by an increased intraluminal pressure (Cole *et al* 1961)

Correlation between the Enterochromaffin Cells and 5 Hydroxytryptamine

By means of the fluorescence method it is possible to determine precisely the EC counts in the intestinal tissue The mast cells which had a similar yellow fluorescence as the EC were easily differentiated by their morphology and location

On the other hand the spectrophotofluorometric method used for the 5 HT determination is specific for all the 5 hydroxyindoles (Bowman *et al* 1955 Weissbach 1961) among which 5 HT is the most important one in the animal tissue The kind of 5 HT precursors and derivatives in the EC is still obscure Obviously their amount in the animal tissues is so low that they have no signification in the EC count and the 5 HT content determinations (Erspamer 1961) It has been pointed out that only in the mouse and the rat the mast cells contain detectable amounts of 5 HT (Benditt *et al* 1955 Parrat and West 1957 Coupland and Riley 1960) According to Keller (1957) the mast cells of the peritoneal cavity in the rat contain 0 346—0 462 × 10^{-6} μg 5 HT/cell. In the present study the mean 5 HT content in one EC of the rat was about 3—4 times as high (1 2 × 10^{-6} μg) Owing to the small number of mast cells and their low 5 HT content it is obvious that the total amount of 5 HT located in these cells is negligible in comparison to that of the EC Besides the present study showed that only traces of 5 HT could be found in the muscular and serous layers of the cow duodenum as earlier reported by Murray and Wyllie (1964) on the human stomach The 5 HT amount present in the platelets and in the blood plasma remaining in the duodenum is very small in comparison to that of the EC (Erspamer 1961)

In the investigations reported in the literature the EC count and the 5 HT content were determined separately only Verity *et al* (1962) used adjacent tissue pieces of the human large bowel However because the EC count and the 5 HT concentration greatly depend on the anatomical and environmental conditions it is necessary to make the determinations from adjacent tissue pieces in order to obtain reliable correlation comparisons

In all the adult mammals investigated there was a highly significant ($P<0\ 001$) correlation between the EC count and the 5 HT content in the duodenum In the sheep cow and pig this correlation differed to some degree from that of the other animals In the newborn and adult guinea pigs the correlation was similar In the rat duodenum the EC density and the 5 HT concentration decreased during development During growth the correlation remained in principal unchanged although also variations were observed In the cow the correlation between the EC and 5 HT was quite similar in the mucous submucous layer and in the whole intestinal wall All these observations strongly support the opinion that the greatest amount of the duodenal 5 HT is located in the EC because the EC and mast cells were the only morphological structures that showed a characteristic yellow fluorescence

Earlier only Koch and Engelhardt (1959) have estimated the 5 HT content in one EC According to them one jejunal EC of the pig contained 2 16 × 10^{-6} μg 5 HT The mean value obtained in this study per one duodenal EC of the pig was about twice as high (5 9 × 10^{-6} μg) but however of the same order of magnitude In the other domestic animals the 5 HT content per EC was quite similar and somewhat larger than in the guinea pig mouse and rabbit Only in the rat the 5 HT content per one EC was distinctly lower (1 2 × 10^{-6} μg) These differences between the various species with respect to the 5 HT content could not be observed in the formaldehyde induced fluorescence or in the argyrophilia but they have obviously some effect on the other staining reactions

If it is assumed that the fluorescent area of the EC is a symmetrical sphere the concentration of cytoplasmic 5 HT in the EC can be calculated This concentration in the species studied varied between 2 4 and 9 7 mg/ml In the electron microscopic pictures the enterochromaffin granules are seen distributed diffusely in the cytoplasm and constitute only a small portion of this cell organ (Christie 1955 Wetzstein *et al* 1962 Toner 1964) It is therefore obvious that the 5 HT concentration in the storage granules is several times as large as the above stated values

IV EXPERIMENTAL STUDIES

EFFECT OF RESERPINE ON THE ENTEROCHROMAFFIN CELLS AND 5 HYDROXYTRYPTAMINE IN THE DUODENUM

Reserpine Experiments

A total number of 45 adult guinea pigs and 77 rats were studied

Reserpine (Serpasil)[R] Ciba AG) was given to the adult animals intraperitoneally and the volume of the injection was adjusted to 4 0 ml with saline A volume of 0 1 ml of reserpine diluted with saline was given to the newborn rats subcutaneously

1st Reserpine Experiment — The guinea pigs received the following doses of reserpine 0.25 mg/kg (5 guinea pigs) 1 0 mg/kg (5) 4 0 mg/kg (5) 30 0 mg/kg (3) All the animals were killed 24 hours after the injection

2nd Reserpine Experiment — 4 0 mg reserpine/kg was given to 15 guinea pigs Five of them were killed 48 five 96 and five 144 hours after the injection

3rd Reserpine Experiment — 2 5 mg reserpine/kg was given to 8 adult rats and 10 0 mg/kg to 7 adult rats All the animal were killed 24 hours after the injection

4th Reserpine Experiment — An injection of 5 0 mg reserpine/kg (7 adult rats) 15 0 mg/kg (4) or 30 0 mg/kg (10) was given twice 48 and 24 hours before killing

5th Reserpine Experiment — 20 0 mg reserpine/kg was given to 10 rats 1 day of age 24 hours before killing

The treatment of the controls was similar to that of the experimental animals with the exception that the injections contained only physiological NaCl Ten adult guinea pigs and 21 adult and 10 two-day old rats were used as controls

Already with the small doses of reserpine the animals showed a typical sedation eyelid ptosis and piloerection With the larger doses a moderate degree of diarrhoea and often haemorrhages in the gastrointestinal tract were observed

The mortality was 50 % in the guinea pigs given the dose of 30 0 mg reserpine/kg about 20 % in the adult rats given the dose of 30 0 mg/kg twice and about 60 % among the newborn rats that received 20 0 mg reserpine/kg

The 5-HT content of the duodenum was determined in all the animals The number of EC was counted in the duodenum of all the animals in groups 1 2 and 5 and in their controls The quantitative estimation of EC was also made for six rats which received the dose of 30 0 mg reserpine/kg

The staining reactions were made in all the animals in groups 1 2 and 3 as well as in six rats which received the dose of 30 0 mg reserpine/kg The formaldehyde induced fluorescence reaction was studied in all the experiments


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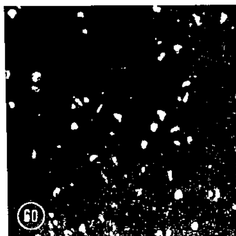


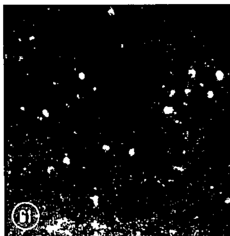
GUINEA PIG

Qualitative Studies

Formaldehyde Induced Fluorescence — Under reserpine action the fluorescent EC exhibited a similar bright yellow colour as the EC of the untreated animals Also the distribution of the EC was similar to that of the controls and the largest patterns were always seen in the basal parts of the mucosa The fluorescence in the mast cells did not weaken essentially under the action of reserpine The greenish fluorescence of the nerve plexus became very weak already after the lowest dose of reserpine

After the doses of 0 25 and 1 0 mg reserpine/kg the EC exhibited quite similar fluorescence properties as those of the controls (Figs 52—54) Still after the dose of 4 0 mg/kg the main bulk of the EC were strongly fluorescent (Fig 55) However the weakly fluorescent EC were then more numerous than in the controls After the treatment with 30 0 mg/kg the general fluorescence intensity of the EC was weak (Figs 60 and 61) Two to six days after the reserpine injection of 4 0 mg/kg no essential difference in the fluorescence intensity of the EC was observed between the experiments and controls

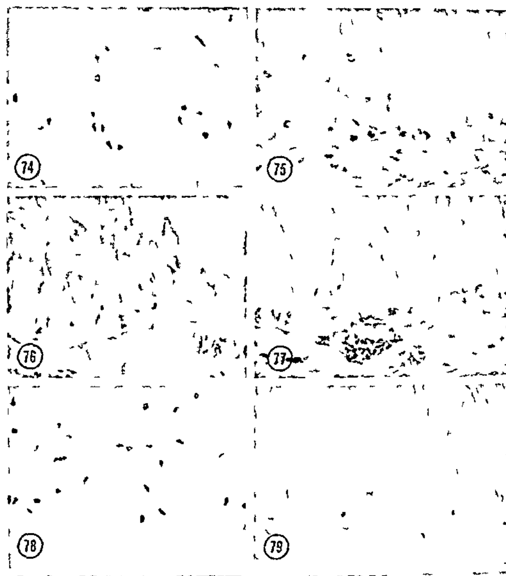




Formaldehyde induced fluorescence in duodenal EC of the guinea pig 24 hours after reserpine treatment × 145
Fig 60 Control
Fig 61 30 0 mg reserpine/kg The fluorescence intensity of the EC is obviously decreased

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Formaldehyde induced fluorescence and argyrophil reactions in duodenal EC of the guinea pig 24 hours after reserpine treatment × 125
Fig 52 Control
Fig 53 0 25 mg reserpine/kg Numerous strongly fluorescent EC are seen
Fig 54 1 0 mg reserpine/kg Numerous strongly fluorescent EC are seen
Fig 55 4 0 mg reserpine/kg Numerous strongly and some weakly fluorescent EC are seen
Fig 56 Control
Fig 57 0 25 mg reserpine/kg Numerous strongly stained EC are seen
Fig 58 1 0 mg reserpine/kg Numerous strongly stained EC are seen
Fig 59 4 0 mg reserpine/kg Numerous strongly stained EC are seen



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81

Argyrophil Reaction — After all the doses of reserpine the argyrophil reaction remained intensively positive in the LC (Figs 56—59 62 and 63)

Other Staining Reactions — *0.25 mg reserpine/kg* In the argentaffin and ferric ferricyanide reactions most of the positive EC were stained as in the controls (Figs 66 67 70 and 71) In the diazo coupling reaction there were numerous weakly stained and some strongly positive EC (Figs 74 and 75) The indophenol reaction showed only some positive EC (Figs 78 and 79)

1.0 mg reserpine/kg Only some strongly stained EC were seen in the argentaffin and ferric ferricyanide reactions (Figs 68 and 72) Most of the EC were not stained specifically In the diazo coupling reaction a few EC stained weakly (Fig 76) The indophenol reaction was very weakly positive only in few EC (Fig 80)

4.0 mg reserpine/kg All the reactions were negative in the EC (Figs 64—65 69 73 77 and 81)

2 days after reserpine treatment (4.0 mg/kg) In the argentaffin and ferric ferricyanide reactions some moderately stained EC were seen but some specimens were negative The diazo coupling reaction showed very few weakly stained EC The indophenol reaction was negative

4 days after reserpine treatment (4.0 mg/kg) The main bulk of the EC were strongly stained in the argentaffin and ferric ferricyanide reactions although several weakly coloured EC were seen In the diazo coupling and especially in the indophenol reaction the number of strongly positive EC was small

6 days after reserpine treatment (4.0 mg/kg) In the argentaffin and ferric ferricyanide reactions the EC were stained as strongly as in the controls In the diazo coupling and especially in the indophenol reaction the number of weakly stained EC was still larger than in the controls

Quantitative Studies

No essential difference in the diameter of the EC was observed in the control and experimental animals Therefore the diameter of 11.7 μ was used for the Floderus formula in all the experimental groups

The decrease in the number of fluorescent EC was quite linear to the logarithm of the reserpine dose (Fig 82) The dose of 30.0 mg reserpine/kg caused an about 57 % decrease in the EC count

Even the lowest dose of reserpine caused a remarkable decrease (36.5 %) in the 5 HT concentration in the duodenum With increasing doses the decrease

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Diazo coupling and indophenol reactions in duodenal EC of the guinea pig 24 hours after reserpine treatment × 125

Fig 74 Diazo coupling reaction Control

Fig 75 Diazo coupling reaction 0.25 mg reserpine/kg Some strongly and numerous weakly stained EC are seen

Fig 76 Diazo coupling reaction 1.0 mg reserpine/kg Only a few specifically stained EC are seen

Fig 77 Diazo coupling reaction 4.0 mg reserpine/kg No specifically stained EC are seen

Fig 78 Indophenol reaction Control

Fig 79 Indophenol reaction 0.25 mg reserpine/kg A few stained EC are seen

Fig 80 Indophenol reaction 1.0 mg reserpine/kg Only very few EC are just visible

Fig 81 Indophenol reaction 4.0 mg reserpine/kg No specifically stained EC are seen

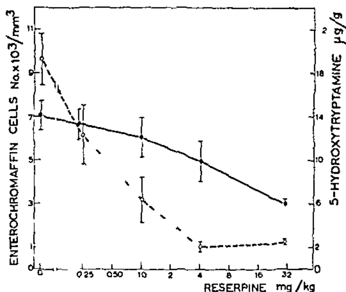


Fig. 82 The logarithmic dose response curve of reserpine on the EC number and the 5 HT concentration in the duodenum of the guinea pig. Ordinate left EC No × 10³/mm³ duodenal tissue. Ordinate right 5-HT concentration µg/g duodenal tissue. Abscissa reserpine dose mg/kg given 24 hours before killing. EC marked —•—•— 5-HT marked —○—○—○—○— Each point is a mean value ± standard deviation

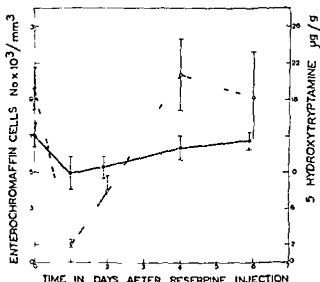


Fig. 83 The effect of reserpine on the EC number and the 5-HT concentration in the duodenum of the guinea pig. Ordinate left EC No × 10³/mm³ duodenal tissue. Ordinate right 5-HT concentration µg/g duodenal tissue. Abscissa time in days after a reserpine dose of 4 0 mg/kg EC marked —•—•— 5 HT marked —○—○—○—○— Each point is a mean value ± standard deviation

was quite linear and the basal mean value (2 1 µg 5 HT/g tissue) was obtained with the dose of 4 0 mg reserpine/kg The dose of 30 0 mg/kg was not able to reduce further the 5 HT concentration as is seen in Fig 82

The reappearance of the fluorescent EC after a single reserpine dose of 4 0 mg/kg is seen in Fig 83 The number of cells reached the control level slowly within six days after the injection On the other hand the 5 HT content increased very rapidly (Fig 83) and the control level was reached within four days

In all the reserpinized guinea pigs the standard deviations in the EC counts were of the same order (about 12 %) as in the controls During the reappearance of 5 HT after reserpine treatment the standard deviations were generally much greater than in the controls

RAT

Qualitative Studies

Formaldehyde Induced Fluorescence — After each dose of reserpine the fluorescent EC were seen distinctly No significant weakening in the fluorescence intensity was observed in the adult rat even after a large dose of reserpine (30 0 mg/kg given twice)

Staining Reactions — After all the doses of reserpine EC were strongly stained in the argyrophil reaction In the argentaffin ferric ferricyanide and diazo coupling reactions there were rather more positive EC than in the controls Because of the random nature of these reactions in the demonstration of the EC in the rat no precise counts were made The indophenol reaction was negative as in the controls

Quantitative Studies

The diameter of the EC determined for the Floderus correction was 9 7 µ in the adult untreated rat 9 8 in the adult rat given 30 0 mg reserpine/kg twice and 9 8 in the untreated and the reserpinized newborn rat

The doses of 2 5 and 10 0 mg reserpine/kg given once or the doses of 5 0 and 15 0 mg/kg given twice were not able to reduce significantly the 5 HT concentration in the duodenum of the rat (Fig 84) However the maximum dose of 30 0 mg/kg given twice caused a considerable decrease in the 5 HT content (50 2 %) which was highly significant ($P < 0\ 001$)

Since the smaller doses of reserpine had no apparent influence on the EC fluorescence and the 5 HT concentration in the duodenum of the rat the EC counts were made only after the highest reserpine dose (30 0 mg/kg given twice) After this treatment the decreace in the number of the fluorescent EC was not significant being about 5 % (Fig 85)

In the newborn rats a very significant decrease (63 8 %) was observed in the 5 HT concentration after a large reserpine dose (20 0 mg/kg) After this treat ment the fluorescence intensity of the EC became distinctly weaker than that in the controls However the cells were still clearly visible and no essential difference was observed in the number of EC (Fig 85)

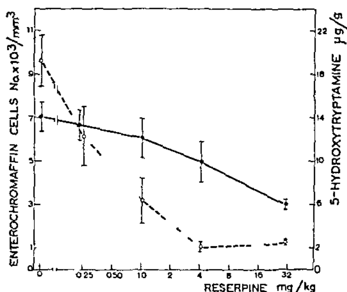


Fig 82 The logarithmic dose response curve of reserpine on the EC number and the 5 HT concentration in the duodenum of the guinea pig Ordinate left EC No × 10³/mm³ duodenal tissue Ordinate right 5-HT concentration μg/g duodenal tissue Abscissa reserpine dose mg/kg given 24 hours before killing EC marked — — — •— — 5 HT marked —o—o—o—o— Each point is a mean value ± standard deviation

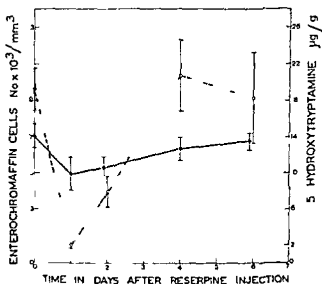


Fig 83 The effect of reserpine on the EC number and the 5-HT concentration in the duodenum of the guinea pig Ordinate left EC No × 10³/mm³ duodenal tissue Ordinate right 5-HT concentration μg/g duodenal tissue Abscissa time in days after a reserpine dose of 4 0 mg/kg EC marked —• • — • 5-HT marked —o—o—o—o— Each point is a mean value ± standard deviation

EFFECT OF CORTISONE ON THE ENTEROCHROMAFFIN CELLS AND 5 HYDROXYTRYPTAMINE IN THE DUODENUM

Material

A total number of 24 newborn rats were studied Cortisone acetate (Adreson N V Organon) was diluted with saline A daily injection of 0 5 mg cortisone in a volume of 0 1 ml was given subcutaneously on 5—6 days The first injections were given at the age of 1 day The controls received 0 1 ml of physiological NaCl daily The body weight of the experiments (5—8 g) did not clearly differ from that of the newborn rats (5—6 g) and was distinctly smaller than that of the controls (14—17 g)

Results

The EC diameter determined for the Floderus correction was 9 8 μ in both the experimental and control animals

On the fluorescence properties size shape granularity and distribution of EC the chronic cortisone treatment had no essential effect. Few similarly fluorescent mast cells were seen This kind of yellow fluorescence was not noticed in the other tissue components

The mean number of fluorescent EC (8 670 EC/mm³ tissue) after cortisone treatment was significantly greater ($P<0$ 001) than that of the controls (5 790/mm³) or that of the newborn rats (5 260/mm³) as is seen in Fig 85 Cortisone injections had a similar effect on the 5 HT concentration The respective mean values were 13 6 μg 5 HT/g tissue in the experiments 7 9 μg in the controls and 10 8 μg in the newborn untreated rats (Fig 85)

DISCUSSION

Reserpine Studies

Pletscher *et al* (1955) found that 5 HT could be liberated from the gastrointestinal tract by reserpine Benditt and Wong (1957) observed that in the argentaffin and diazo coupling reactions the EC decrease and the 5 HT release were quite linear to the logarithm of the reserpine dose in the duodenum of the guinea pig They also observed the coincident reappearance of the EC and 5 HT Later it was demonstrated that all the staining reactions with the exception of the argyrophil reaction became negative in the EC after reserpine treatment (Zbinden *et al* 1957 Lillie *et al* 1959 Prellwitz 1959)

In the present study the disappearance and reappearance of the EC and 5 HT was confirmed in the duodenum of the guinea pig after reserpine treatment It was further noticed that reserpine first abolished the diazo coupling and indophenol reactions in the EC Already the smallest dose of reserpine turned almost all EC indophenol negative The diazo coupling and indophenol reactions also reappeared in the EC more slowly than the argentaffin and ferric ferricyanide reactions On the other hand even after the largest dose of reserpine the argyrophil granules were as strongly positive as in the controls indicating that the argyrophil reaction must be very sensitive or that it is rendered positive by other properties of the EC than those causing the other staining reactions These

experimental results as well as the results obtained with untreated animals indicate that the staining of the EC does not reflect the duodenal 5 HT concentration alone and that the diazo coupling and indophenol reactions demonstrate the EC less effectively than the argentaffin and ferric ferricyanide reactions

The effect of reserpine on the fluorescence reaction in the EC has not been investigated earlier The present study showed that even a relatively large dose of reserpine (0 25—4 0 mg/kg) was not able to reduce essentially the fluorescence intensity of the EC However after the largest dose (30 0 mg/kg) the average fluorescence intensity of the EC became distinctly weaker The mean concentrations of 5 HT in one EC were 2.9 $\times 10^{-6}$ (control) 1 9 $\times 10^{-6}$ (0 25 mg reserpine/kg) 1 0 $\times 10^{-6}$ (1 0 mg/kg) 0 4 $\times 10^{-6}$ (4 0 mg/kg) and 0 7 $\times 10^{-6}$ (30 0 mg/kg) Two four and six days after reserpine treatment (4 0 mg/kg) the mean 5 HT contents of the EC were 1 6 3 5 and 2 6 $\times 10^{-6}$ µg These results suggest that the fluorescence intensity does not reflect the 5 HT concentration of the EC in a linear manner

The number of fluorescent EC after reserpine treatment was linear to the logarithm of the reserpine dose After the largest dose however 43 % of the EC were still seen This indicates the high sensitivity of the formaldehyde induced fluorescence in demonstrating EC in comparison to the staining reactions In contrast to the staining reactions the disappearance and reappearance of the EC and of 5-HT after reserpine treatment occurred strikingly concomitantly in the fluorescence reaction

The slight effect of large doses of reserpine on the 5 HT concentration in the rat intestine (Erspamer 1956 West 1958 Karki and Paasonen 1959) was also observed in the present study Twentyfour hours after the reserpine injections (2 5—15 0 mg/kg) the 5 HT level in the duodenum remained quite similar to that of the controls Only after the dose of 30 0 mg/kg a distinct decrease of 50 2 % was observed in the adult rat In the newborn rat 5 HT was more easily liberated and a single dose of 20 0 mg/kg already caused a 63 8 % release of 5 HT Obviously the traces of the amines are bound more tightly in the EC granules or the chemical binding mechanism is dissimilar to that of the main bulk of the amines which probably explains the different reserpine effect on 5 HT in the guinea pig and newborn rat in comparison to the adult rat The synthesis of 5-HT can occur also more rapidly in the rat than in the guinea pig

In the rat the otherwise weak staining reactions in the EC did not disappear after reserpine treatment (Marks *et al* 1958 Ghiringhelli and Mira 1959) The present study lends further support to these observations Even the dose of 30 0 mg/kg did not abolish the staining of EC in the rat duodenum On the contrary after the smaller doses the stained EC were more numerous than in the controls Reserpine had no effect on the fluorescence properties of the EC in the adult rat whereas in the newborn rat the fluorescence intensity weakened to some degree after reserpine treatment indicating dissimilar attitudes of the EC in these age groups However under reserpine action the EC counts remained at both ages quite similar to those of the controls

It was reported by Zbinden *et al* (1957) that reserpine had a distinct effect on the staining reactions in the EC and on the 5 HT concentration in the pyloric part of the rabbit stomach but no effect in the fundic part Also in various organs and species the EC behaved dissimilarly towards reserpine (Erspamer 1961)

Both these observations as well as the dissimilar attitude of the [illegible] to reserpine in the guinea pig and in the adult and newborn rat [illegible] ences in the chemical nature or physiological function of the en[illegible] granules in the various gastrointestinal parts age groups [illegible]

Cortisone Studies

According to West (1958) small amounts of cortis[illegible] given during two weeks had no effect on the duodenal 5 HT content in the adult rat Later however Telford and West (1960) demonstrated that cortisone and fludrocortisone reduced 5 HT in the small intestine of the rat The effect [illegible] one on the duodenal EC has not been studied earlier

It was observed in the present study that in comparison to the controls the chronic cortisone treatment caused a significant increase of the duodenal EC density (50 %) and the 5 HT concentration (71 %) in the newborn rat Also in comparison to the body weight which was of a similar size class in the experiments and untreated newborn rats the duodenal EC density and the 5-HT content increased during cortisone treatment Possibly cortisone accelerates in some way the functional maturity of EC and causes the increase of the EC density and the 5 HT content (Moog and Richardson 1955) Also the physiological function of the intestine is checked to some degree because of the lethargic condition of the animals and 5 HT is then obviously accumulated in EC. On the fluorescence properties of the EC cortisone had no effect

The cortisone dose however was so great that its effect on the EC and 5 HT was obviously quite unspecific and to some extent due to the very striking dehydration in the body Therefore it is difficult to speak of a specific effect of cortisone on the EC and 5 HT Lempinen (1964) found that cortisone specifically prevented the disappearance of the extra adrenal chromaffin tissue and even created new chromaffin cells These observations give reason for the assumption that the extra adrenal chromaffin tissue and EC have dissimilar functions and are not regulated by the same mechanisms

In the cortisone experiments the correlation between the EC and 5-HT remained quite similar to that in the controls This supports Erspamer s (1958) hypothesis that 5 HT is concentrated in the EC

SUMMARY

1 *Histochemical Reactions of Enterochromaffin Cells*

In the duodenal EC of the sheep, cow, pig, horse, guinea pig, mouse, rat and rabbit the formaldehyde induced fluorescence and five staining reactions were studied. The fluorescence method was observed to be the most specific and sensitive one. Among the staining reactions the argyrophil method, in spite of its poor specificity, coloured the EC most effectively.

Most of the EC were found in the mucous layer, very few of them in the submucosa and none of them in the other layers of the duodenum.

The *formaldehyde induced fluorescence* was intense and yellow in the EC of all species. The EC of all the animals exhibited also an intense *argyrophilia*. When the fluorescence and argyrophilia were consecutively seen in the same sections it was observed that all the fluorescent EC were also argyrophil. The *argentaffin* reaction stained all the EC in the sheep, cow, pig and guinea pig. In the duodenum of the other species only several or some of the fluorescent EC were argentaffin.

The staining ability of the EC towards the *ferric ferricyanide, diazo coupling* and *indophenol* reactions varied considerably. Only in the pig and guinea pig were these three reactions positive in all the fluorescent EC. In the other species these reactions were in general moderate or weak and only some EC were stained. The duodenal EC of the horse, mouse and rat were entirely indophenol negative.

The sections examined by both the fluorescence and the staining reactions showed no stained EC that did not have the yellow fluorescence properties.

The EC of none of the species investigated showed autofluorescence in the freeze dried duodenum.

2 *Enterochromaffin Cells and 5-Hydroxytryptamine in the Mammalian Duodenum*

The number of fluorescent EC (No./mm³ duodenal tissue) and the 5-HT concentration (µg/g duodenal tissue) were determined from adjacent duodenal tissue pieces taken near the pylorus of the above mentioned eight adult mammalian species, as well as from the developing duodenum of the guinea pig and the rat.

Numerous EC were observed in the guinea pig and sheep, a few in the horse and moderate amounts in the other animals. The 5-HT concentration was high in the duodenum of the sheep and guinea pig and markedly low in the horse and rat, whereas all the other animals showed moderate amounts of 5-HT.

The correlation between the EC number and the 5-HT concentration was highly significant ($P < 0.001$).

In the newborn guinea pig the EC count and the 5 HT concentration were similar to those of the adults On the other hand in the newborn rat both the EC number and the 5 HT content were significantly above the adult level which was reached at the age of 24—32 days

The mean 5 HT contents of the EC were greatly variable the standard deviations being 12 3—47 1 % The highest values were observed in the pig and in the other domestic animals and the lowest value in the rat.

3 *Experimental Studies*

The effect of reserpine on the fluorescence and staining reactions as well as on the EC number and the 5 HT concentration was studied in the duodenum of the guinea pig and rat The effect of cortisone on the duodenal EC and 5 HT was investigated only in the newborn rat

Reserpine — In the guinea pig the argyrophilia of the EC remained uninfluenced under reserpine action Reserpine abolished more effectively the diazo coupling and indophenol reactions than the argentaffin and ferric ferricyanide reactions but had a weak effect on the formaldehyde induced fluorescence The disappearance and reappearance of the staining reactions was linear both to the logarithm of the reserpine dose and to the time after the injections In the fluorescence reaction the EC number and the 5 HT content decreased and increased concomitantly but on the other hand linearly in the staining reactions

In the adult rat reserpine caused no essential histochemical alterations in the EC whereas in the newborn rat the formaldehyde induced fluorescence reaction weakened under reserpine action The largest doses reduced the 5 HT concentration significantly without causing any alterations in the EC number of the adult or the newborn rat

Cortisone —The chronic cortisone treatment had a significantly increasing effect on both the EC number and the 5 HT concentration in the newborn rat The fluorescence properties of the EC remained uninfluenced

4 *Conclusions*

The similar size shape coarse granularity yellow fluorescence and strong argyrophilia of all the EC and their identical distribution in the intestinal wall of the mammalian species investigated suggest that the enterochromaffin system is morphologically and functionally similar in the different species and that there is only one principal type of EC in adult mammals

Towards the other staining reactions the enterochromaffin granules showed a variable affinity and there were also species differences The sensitivity of the staining reactions in demonstrating the EC decreased in the following order argyrophil argentaffin ferric ferricyanide diazo coupling and indophenol reaction In the guinea pig reserpine had a marked influence on these staining reactions a weak effect on the fluorescence and no effect on the argyrophil properties

The studies of the EC count and the 5 HT concentration in the duodenum of the adult mammals in the developing duodenum of the guinea pig and the rat in the different layers of the cow duodenum and in the cortisone experiments support the hypothesis that the EC number and the 5 HT concentration are in a direct correlation to each other in the mammalian duodenum The histochemical and pharmacological studies tend also to show that most of the duodenal 5 HT is located in the EC.

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PIGMENT MIGRATION, LIGHT SCREENING AND RECEPTOR SENSITIVITY IN THE COMPOUND EYE OF NOCTURNAL LEPIDOPTERA

BY

GUNNAR HÖGLUND

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CONTENTS

INTRODUCTION

The amount of light reaching the light sensitive parts of the photoreceptor cells may be regulated by several mechanisms in the eye These mechanisms include movements of the iris and its appendages, the exposure or covering of light reflecting layers, and movements of the receptors or of retinal "screening' pigments

Pigment movements occur together with other regulatory mechanisms in many animal groups such as birds reptiles amphibians and molluscs In some groups e g in mammals pigment movements are usually considered not to occur while in the eyes of other forms such as the compound eye of some arthropods pigment migration is regarded as the only mechanism regulating the intensity of light reaching the receptors (See Parker 1932 and Duke Elder 1958)

It has recently been shown that the retinal sensitivity varies with the pigment position in arthropod mollusc and vertebrate eyes In the compound eye of night moths the sensitivity increase during dark adaptation often proceeds in two phases The second of these phases occurs in parallel with the outward pigment movement (Bernhard and Ottoson 1960 a 1960 b 1964 Bernhard Hoglund and Ottoson 1963, Post and Goldsmith 1965) Experimental results indicate that movements of the proximal pigment influence the sensitivity of the compound eye in some crustaceans (de Bruin and Crisp 1957) In the molluscs *Octopus* and *Ommastrephes* the sensitivity increase during dark adaptation has been related to pigment migration (Byzov Orlov and Utina 1962) Dodt and Jessen (1961) have reported that in the frog the pigment migration occurring when the eye is exposed to an adapting light causes a threshold decrease of more than one log unit Back Donner and Reuter (1965) found that the amount of rhodopsin bleached by exposing the frog eye to a standard light varies with the pigment position The bleaching efficiency increases when the pigment retracts from the rod outer segments Back Donner and Reuter concluded that the migration of the pigment from the dark adapted to the light adapted position reduces the effective intensity of the light to 1/3

The investigations mentioned above provide evidence that the photoreceptor sensitivity is influenced by the pigment position The experiments described in the present paper were undertaken in an attempt to study the relation between the receptor light sensitivity and the variations in screening effect dur

ing pigment movements The experiments were done on the compound eye of night moths (mainly *Celerio euphorbiae* and *Deilephila elpenor*) These species were chosen because both in the light adapted and in the dark adapted state the migrating accessory pigment is located between the cornea and the retinular cell rhabdom which is presumed to be the primary site of photoreception (see Goldsmith 1964) The anatomical separation between the migrating pigment and the rhabdoms was taken advantage of to develop an experimental technique which permitted the receptor sensitivity to be determined not only by light transmitted through the cornea and the pigment but also by exposing the receptors directly to a test light The experiments reported in part I were done to find out if variations in the glow (page 16) can be used as an index of accessory pigment movements The position of the pigment near the basement membrane in light adapted and dark adapted eyes was also determined This pigment is believed to be located in the basal pigment cells (Stefanowska 1890, Yagi and Koyama 1963) Part II describes studies of receptor threshold and screening effect during pigment movements in dark adapted eyes In the experiments described in part III dark adapted eyes were exposed to adapting illuminations of varying intensities and durations In some experiments the pigment remained in a distal position during the illumination while in other experiments the pigment migrated in the proximal direction The relation between the receptor threshold and the pigment position under these experimental conditions was studied

The principal results have been described in a short previous report (Höglund 1963 b) A communication has also been presented at the Symposium on the Functional Organization of the Compound Eye held in Stockholm in 1965 (Höglund 1966)

MATERIAL AND METHODS

Material The experiments were performed on compound eyes from adult specimens of the moths *Celerio euphorbiae* and *Deilephila elpenor* (Sphingidae) A few experiments were also done on *Noctua pronuba* (Noctuidae) The species were identified by the names given in the list of Lepidoptera published by Heslop (1959, 1960)

The specimens of *Noctua pronuba* were caught wild and the Sphingidae were raised in captivity The experiments on the specimens caught wild were done within 2 days after capture Moths raised from pupae were used for experiments 1 to 7 days after their emergence The specimens were usually kept in darkness for 12 hours before the experiments, the minimum time being 4 hours, and the maximum time 24 hours The experiments were done at room temperature 20 to 26°C Experiments in which the eyes were illuminated through a glass fiber were done on 310 eyes These experiments were performed on *Celerio euphorbiae* and *Deilephila elpenor* All eyes were of the normal type containing dark pigment granules Experiments were done both at night time and daytime The results were not influenced by the time of day when the experiments were done

Dissection The eye was excised in such a way that the chitinous ocular ridge or sclerite ring (see Snodgrass 1935 and Yagi and Koyama 1963) remained intact The optic lobe was then removed except for the lamina (see Bullock and Horridge 1965) and, in some eyes part of the medulla The parts of the optic lobe that were not removed were severed with a fine needle The dissection was performed under dim white light Throughout the dissection the eyes exhibited a maximal glow (page 16) If the glow diminished during the dissection the eye was discarded

In some experiments the cornea crystalline cones and distal pigments were removed prior to the electrophysiological measurements The cornea was cut around the margin of the eye and was removed from the eye together with the crystalline cones and the distal pigments which adhered to the cornea The dissection was made when the glow was maximal so that the retinular rhabdoms were left intact Microscopical inspection showed that the distal pigment granules were removed from all parts of the eye except in those ommatidia immediately adjacent to the sclerite

Light stimulation During the electrophysiological experiments the eye was

kept in a light tight box. The adapting and test lights were obtained from a tungsten lamp mounted in a Zeiss dissecting microscope (Zeiss tungsten lamp 390158 operated at 7.2 V AC from a stabilized power source, color temperature 2900 to 3100°K). The unattenuated light intensity measured at the cornea was about 35000 lux for both the adapting and test lights. In this paper the relative intensities are given in logarithmic units with the unattenuated intensity as reference level. The unattenuated intensity is referred to as log relative intensity 0 ($\log_{10}1$), an intensity attenuated to 1/10 as log relative intensity −1 ($\log_{10}0.1$) etc. The beam of light from the microscope was split into two beams, one beam being used as the adapting light and the other as the test light. The area illuminated by the adapting light was identical with that illuminated by the test light. This area was many times larger than the surface of the eye. The intensities of the adapting and test lights were independently controlled by neutral density filters (Bausch and Lomb 31 34 38 or Schott NG 3) and a heat filter (Balzer Calflex B 1 K 1 or B 1 K 2). In addition, the intensity of the test light could be gradually attenuated by a neutral density wedge (Kodak). Control experiments showed that the threshold value did not change if, in the course of an experiment, the two optical paths were interchanged. The positioning of the eye in the center of the illuminated field was not critical since the eye could be displaced by more than its own diameter without any measurable change in threshold value.

The duration of the test flashes was 0.2 sec. This duration permitted the amplitude of the retinal potential change to be measured, but was short enough not to cause any measurable change in the threshold value. The duration of the test illumination was controlled by a mechanical shutter (Praktika). The exposure time was regularly checked by means of a photocell connected to the oscilloscope. The duration of the adapting illumination was controlled by a manually operated shutter.

Test flashes were also conducted to the eye through a coated glass fiber (American Optical Company, core glass refractive index = 1.62, coating glass refractive index = 1.52). The fiber had a total diameter of 75 μm and a total length of about 15 cm. The fiber was inserted into the eye by a micromanipulator, the depth being determined by a micrometer gauge. Illumination was provided by a tungsten lamp (Osram 8032, operated at 12 V AC, color temperature 2900 to 3000° K, or 18 V AC, color temperature 3300 to 3400° K. The operating voltage was provided by a stabilized power source.) When one end of the glass fiber was exposed to the unattenuated light from the tungsten lamp the luminous flux emitted from the other end of the fiber was 25 μlm (12 V) or 140 μlm (18 V). The maximal luminous intensity was 3 μcd (12 V) or 17 μcd (18 V). The unattenuated light intensity is referred

to as log relative intensity 0 The intensity of the light entering the glass fiber was attenuated by filters and a wedge of the same specifications as mentioned above The duration of the test flashes 0 2 sec, was controlled by a mechanical shutter (Ibsor) The spectral transmittance of the fiber was measured in a spectrophotometer (Spectronic) According to these measurements the maximal deviation from perfect neutrality was about ±20% for wavelengths between 450 and 700 nm The transmittance was less for wavelengths shorter than 450 nm Control experiments using fibers that had been given an extra coating of black lacquer showed that the experimental results were not influenced by light escaping through the glass fiber walls

Figure 1 A shows a photograph of an eye with a fiber penetrating the cornea Figure 1 B shows a histological section of an eye with a fiber drawn to scale It is seen that the diameter of the fiber is about 3 times the diameter of an ommatidium Inspection of sliced fresh eyes showed that in spite of the small diameter of the fiber, most of the ommatidia within the eye were illuminated by the light coming from the fiber This may partly be due to the fact that the beam of light emitted from the fiber was very divergent It may also be due to reflection and scatter by retinal structures such as the tracheae and retinular cells

Figure 2 is a schematic drawing indicating various positions of the fiber tip within the eye In most experiments the fiber was inserted through the cornea and the tip was placed between the accessory pigment and the pigment near the basement membrane (Fig 2 A and B) In some experiments the fiber was inserted through the basement membrane and the tip was located proximal to the accessory pigment (Fig 2 C) and in other experiments the tip was located just below the inner surface of the cornea (Fig 2 D)

Recording of potential changes The eye was placed on a filter paper moistened with a saline solution (Ephrussi and Beadle 1936) The eye was fixed to a cork plate by means of pins through the chitin surrounding the eye The recording electrode was an Ag AgCl wire (diameter 50 or 100 μm) the tip of which was placed just below the cornea Another Ag AgCl wire serving as the reference electrode was placed in contact with the moistened filter paper and connected to ground Eyes from which the cornea and distal pigments had been removed were placed in a moist chamber during the experiment and the recording electrode was placed in contact with the exposed retinular cells

The electrodes were connected via a cathode follower to a DC preamplifier (Grass P6 frequency response 0 to 10000 Hz) and a cathode ray oscilloscope (Tektronix 502) In most experiments a capacitor was inserted between the preamplifier and the oscilloscope The resulting coupling time constant of 1 0 sec was sufficiently long not to alter significantly the form of the potential

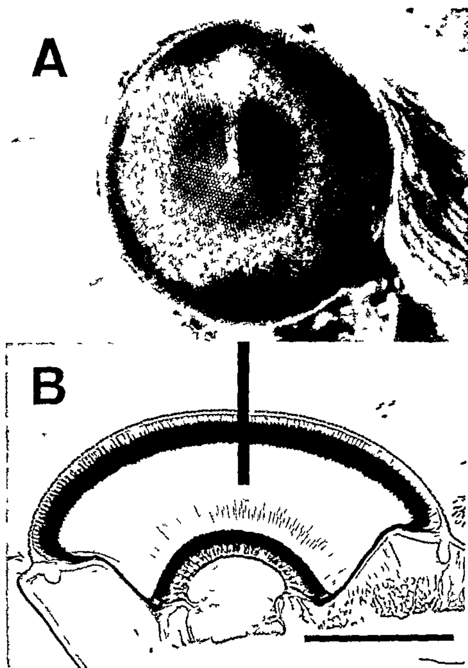


Fig 1 A *compound eye of night moth (Noctua pronuba)* with light conducting glass fiber penetrating through cornea B histological section of eye *(Celerio euphorbia)* showing position of glass fiber Fiber tip located between accessory pigment and pigment near basement membrane Fiber diameter (75 μm) drawn to scale in Fig 1B Horizontal bar 1 mm

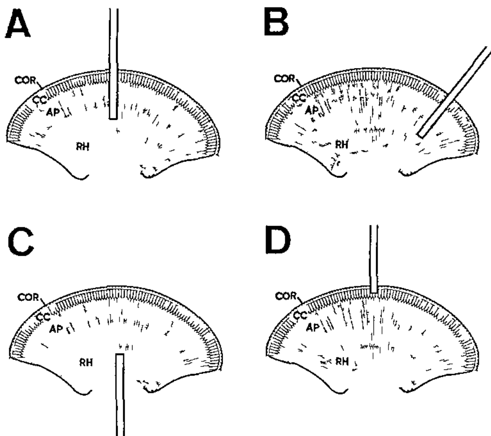


Fig 2 Schematic drawing showing fiber tip at different positions within the eye A fiber inserted through cornea in central part of eye and tip located between accessory pigment and pigment near basement membrane B fiber inserted near margin of eye C fiber inserted through retinal basement membrane D fiber tip located just below inner surface of cornea COR = cornea CC = crystalline cones AP = accessory pigment RH = rhabdoms

change occurring in response to a 0 2 sec light flash Some experiments were also done with direct coupling

The electrodes were coated with black lacquer except at the tip Tests for photoelectric potential changes caused by illumination of the electrodes were performed periodically No such potential changes were recorded at the light intensities used in this investigation

Determination of threshold response The retinal potential change chosen as threshold response was generally 40 or 60 μV (in some experiments 80 μV) The relative light intensity eliciting this potential change is referred to as T when the eye was illuminated in the conventional manner and t when the eye

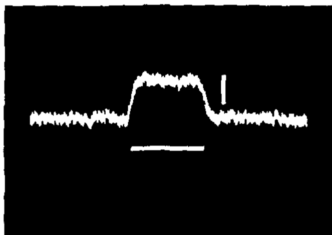


Fig 3 Response of eye to light stimulation of threshold intensity Vertical bar 50 μV Horizontal bar 200 msec Upward deflection indicates negativity of retinal el ctrode

was illuminated through the glass fiber irrespective of the po ition of the fiber tip within the eye Stimulation of the eye by light passing through the ommatidial dioptric media is called transpigment stimulation and the threshold intensity using this stimulation is the transpigment threshold Stimulation through a fiber the tip of which was located proximal to the accessory pigment is referred to as infrapigment stimulation and the threshold as the infrapigment threshold

The potential change in response to a 0 2 sec test flash is shown in Fig 3 The electrode inserted through the cornea became negative with respect to the reference electrode and the negativity was maintained until the end of the flash The potential change was the same whether the eye was exposed to trans pigment or infrapigment light stimulation Regardless of the pigment position the amplitude of the initial transient component of the threshold response did not exceed the amplitude of the sustained component by more than about 10 to 15 μV at the adapting light intensities used in this study

In darkness and at the adapting light intensities used in the electrophysiological experiments (not stronger than log relative intensity —1) an unattenuated test light usually elicited a response the amplitude of which was at least 20 times larger than the value chosen as the threshold response The only exception was that when the eye was exposed to an adapting light intensity of log relative intensity —1 with the accessory pigment situated distally the response elicited by the unattenuated test light was usually 2 to 5 times greater than the amplitude chosen as threshold

In darkness and at the adapting light intensities used in this study a change in transpigment or infrapigment test light intensity by 0 2 log unit caused a change in response amplitude of about 20 μV or more A response of 20 μV was usually the smallest response that could be clearly separated from the noise The accuracy of the measurements of T and t was therefore estimated to be about $\pm$0 2 log unit The minimum interval between test flashes was about 0 5 min except in some experiments in which test flashes were applied at 0 1 min intervals during the first minute of exposure to an adapting light Control experiments showed that at these frequencies the test stimuli did not cause any measurable change in T or t

Recording of glow When the eyes were exposed to an adapting light this light was also used for observing the size of the glow (page 16) The glow in eyes that were not exposed to an adapting light was observed under a dim white light for about 5 sec immediately after the threshold determinations Control experiments showed that this illumination did not change the size of the glow nor did it change the values for T and t when tested 0 5 min after the observations of the glow

Photographs of some eyes were taken with a microscope camera (Zeiss Aufsetz Kamera) The light source was provided by an electronic flash (Zeiss)

Histological procedures The eyes were kept in Bouin—Duboscq—Brasil s fixation solution (see e g Romeis 1948 or Gatenby and Beams 1950) for 12 to 16 hours They were then successively transferred into 80 and 96 % ethanol Bolcek s solution (see e g Romeis 1948 or Gatenby and Beams 1950), 96 and 99 % ethanol and a mixture of ethanol and anhydrous ether The eyes were embedded in celloidin The unstained sections (about 30 μm thick) were mounted in canada balsam The position of the pigment borders in the histological sections was measured at 0°, $\pm$15°, $\pm$30° and $\pm$45° from the central ommatidia The pigment position is given as the mean of these 7 measurements

The following procedure was used to relate glow size to pigment position Excised dark adapted eyes usually with part of the optic lobe left intact were kept in glass containers and the entire corneal surface was inspected at intervals through the optical system used for observation of the glow during the electrophysiological experiments One series of eyes was kept in darkness The eyes in which the glow had been maximal (page 16) in all parts of the eye for 15 to 30 min were submerged in Bouin—Duboscq—Brasil s fixation solution and left in darkness Another series of dark adapted eyes was exposed to weak light for 20 to 30 min The eyes exhibiting minimal glow (page 16) in all parts of the eye were exposed to the same light for another 20 min The eyes in which the glow remained minimal were submerged in Bouin—Duboscq—Brasil s fixation solution and kept in the same illumination

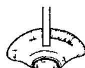
Measurements on fresh eyes In one series of experiments the central part of the eye was sliced with a razor blade at right angles to the corneal surface The eye was placed on a glass cover slip and the distance between the corneal surface and the distal border of the pigment near the basement membrane was measured with an ocular micrometer through a dissecting microscope The results of these measurements are summarized in Table I The results of the same measurements from histological sections (also given in Table I) indicate that the histological procedure caused a shrinkage of about 10 to 15% both in *Celerio euphorbiae* and *Deilephila elpenor* This shrinkage was taken into account when the position of the fiber tip was determined from histological sections

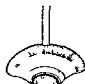
TABLE I Distance (μm) between distal surface of cornea and pigment near basement membrane in fresh eyes and in histological sections Mean values and standard deviation (S D) Number of eyes = n

Species		Fresh eyes	Histological sections
Celerio euphorbiae	Mean	961	844
	S D	45	55
	n	32	116
Deilephila elpenor	Mean	868	780
	S D	69	47
	n	20	99

Symbols used in the illustrations In the illustrations symbols are used which indicate the position of the glass fiber tip within the eye and the size of the glow It is also indicated in the illustrations whether the eyes were kept in darkness or exposed to an adapting light The symbols are explained on page 15


No fiber used


Fiber inserted through cornea and tip located between accessory pigment and pigment near basement membrane


Fiber tip located just below inner surface of cornea


Fiber inserted through retinal basement membrane and tip located between accessory pigment and pigment near basement membrane


Distal pigments removed together with cornea


Maximal glow


Small glow


No glow


Threshold T


Threshold t


Eye kept in darkness


Eye exposed to adapting light


Neutral density filter substituting for pigment

I Pigment migration and "glow"

It is well known that the compound eyes of nocturnal moths and other arthropods reflect light when the eye is dark adapted The reflected light is seen as a bright spot (Leydig 1864), usually referred to as the 'glow', in the part of the eye directed towards the observer (see reviews by Parker 1932 and Goldsmith 1964) The light is reflected from structures within the eye in moths probably mainly from the tracheae (Exner 1891, Merker 1929, Yagi and Koyama 1963) However, according to Day (1941) it is uncertain whether or not the tracheae contribute to the reflection The glow gradually disappears when the eye is illuminated and reappears after the eye is transferred to darkness again

Several authors (Exner 1891, Kiesel 1894, von Frisch 1908, Demoll 1917, Merker 1929 Horstmann 1935, Höglund 1963 a, Yagi and Koyama 1963) have reported that movements of the accessory pigment are associated with changes in the size of the glow However, Parker (1932) has pointed out that glow changes in compound eyes may be determined not only by movements of the accessory pigment but also by movements of pigment located proximally in the eye (see also Goldsmith 1964), and Day (1941) reported that in the compound eye of the moth *Ephestia* the glow rapidly disappears with almost imperceptible changes in the position of the accessory pigment cells Changes in the size of the glow may therefore be associated with movements of the accessory pigment in some species but not in others

This section of the paper reports experiments undertaken to study whether or not glow changes may be used as an index of pigment movements in *Celerio euphorbiae Deilephila elpenor* and *Noctua pronuba*

The anatomy of the eye in *Celerio euphorbiae* has been described by Bugnion and Popoff (1914) and the pigment movements by Stefanowska (1890) A recent general description of the compound eye in the Sphingidae and Noctuidae is given by Yagi and Koyama (1963)

RESULTS

Figure 4 shows a compound eye with no glow (A), with a small glow (B), and with a maximal glow (C) The typical pigment position in an eye from *Celerio*

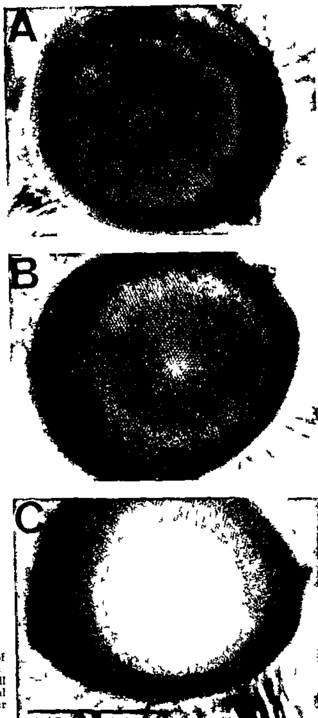


Fig 4 Compound eye of night moth (*Noctua pronuba*) with no glow (A) small glow (B) and maximal glow (C) Horizontal bar 1 mm

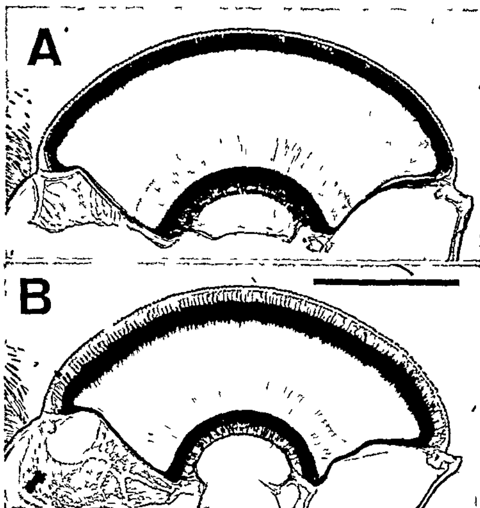


Fig 5 Position of light screening accessory pigment in eyes (*Celerio euphorbiae*) exhibiting maximal glow (A) and minimal glow (B) Note that in eye with minimal glow pigment is located more proximally than in eye with maximal glow Horizontal bar 1 mm

or *Deilephila* with a maximal glow is illustrated in Fig 5 A The accessory pigment is situated around the crystalline cones with the distal pigment border located immediately below the inner corneal surface, and the proximal border almost at the level of the proximal ends of the crystalline cones Figure 5 B illustrates the typical pigment position in an eye exhibiting a minimal glow, i e a glow which is just visible as a minute spot in the center of the area under inspection The distal pigment border is seen to surround the crystalline cones, and the proximal border is located around the distal part of the retinular cytoplasmic processes

Values for the distances between the corneal surface and the distal and proximal borders of the accessory pigment in eyes from *Celerio euphorbiae* and *Deilephila elpenor* with maximal and minimal glow are given in Table II A statistical ' rank sum test (see e g Dixon and Massey 1957) showed a highly significant difference ($p<0\ 001$) between the pigment position in eyes exhibiting maximal and minimal glow Sections were also made of eyes from *Noctua pronuba* In this species, as in *Celerio* and *Deilephila* the pigment was situated more proximally in eyes with minimal glow compared to eyes with maximal glow The results show that in the species investigated a diminution of the glow from maximal to minimal is associated with migration of the accessory pigment in the proximal direction and *vice versa* (see also Hoglund 1963 a)

TABLE II Distance (μm) from proximal surface of cornea to proximal and distal borders of accessory pigment in histological sections of eyes exhibiting maximal or minimal glow Median values and range Number of eyes = n

Species	Glow size		Proximal surface of cornea to		
			Distal pigment border	Proximal pigment border	Distal end of rhabdoms
Celerio euphorbiae	Maximal	Median	0	132	611
		Range	0—19	103—190	499—727
		n	22	22	22
	Minimal	Median	38	219	599
		Range	0—106	136—297	461—724
		n	94	94	94
Deilephila elpenor	Maximal	Median	0	133	555
		Range	0—13	89—165	500—600
		n	26	26	26
	Minimal	Median	74	223	576
		Range	9—139	141—290	441—648
		n	73	73	73

It should be noted that in eyes that have been adapted to a bright light both the proximal and distal borders of the accessory pigment are located considerably more proximally than in eyes exhibiting a minimal glow (see Fig 13 D page 35)

In some species of night moths the position of the pigment located near the basement membrane varies with the adapting light intensity (Tuurala 1954) In these eyes the pigment migrates in the proximal direction in darkness, and in the distal direction when the eye is illuminated Such pigment movements

have been reported to occur in *Celerio euphorbiae* (Stefanowska 1890) The magnitude of the pigment movements was estimated by measurements of the pigment position in dark adapted eyes and in eyes that had been exposed to the unattenuated adapting light (log relative intensity 0) for more than 30 min The mean distance between the basement membrane and the distal border of the pigment in dark adapted and light adapted eyes is given in Table III The results show that movements of the pigment near the basement membrane if they occur at all do not exceed a few μm at the light intensities used in this study

TABLE III Distance (μm) from basement membrane to distal border of pigment near basement membrane in histological sections of dark adapted and light adapted eyes Mean values and standard deviation (S D) Number of eyes = n

Species		Dark adapted	Light adapted
Celerio euphorbiae	Mean	19	20
	S D	1 4	1 9
	n	14	19
Deilephila elpenor	Mean	16	17
	S D	1 5	1 1
	n	15	20

DISCUSSION

The present results are in agreement with the conclusions reached by most earlier investigators (Exner 1891, Kiesel 1894 von Frisch 1908, Demoll 1917, Merker 1929 Horstmann 1935, Yagi and Koyama 1963) that the size of the glow is related to the position of the accessory pigment but are not in accordance with the observation by Day (1941) in experiments on *Ephestia* that the glow may disappear with almost imperceptible changes in position of the pigment cells This discrepancy indicates that apart from the possibility of species variation the results may be influenced by the experimental technique Some earlier investigators have used variations in the size of the glow as an index of accessory pigment movements without correlating the size of the glow to the position of the pigment in histological sections However, Kiesel (1894) and Horstmann (1935) reported that the glow disappears before the pigment has reached the extreme proximal position Yagi and Koyama (1963) reported that the diminution of the glow occurs when the accessory pigment passes the proximal end of the crystalline cones A schematic illustration shows a minute

glow spot when the pigment is surrounding the proximal part of the crystalline cone The present results are consistent with those described by Yagi and Koyama

It is unlikely that movements of the pigment near the basement membrane a few μm could significantly change the reflection of light by the tracheae and thereby cause any variation in glow, since the tracheae extend almost to the proximal end of the rhabdom (Bugnion and Popoff 1914 Yagi and Koyama 1963) The main cause for the glow changes seems to be variations in the light screening by the accessory pigment (Exner 1891)

Edwards (1964) has suggested that glow changes observed in pigment free eyes of the moth *Halisidota* are caused by movements of the accessory pigment cell nuclei Even if movements of these nuclei contribute to the glow changes also in normal eyes the present experiments show that variations in glow are associated with movements of the accessory pigment It should be noted how ever that the glow disappears before the pigment has moved to its most proximal position Pigment movements can therefore occur in the absence of glow The retinular cell nuclei are not mobile in the Sphingidae (Yagi and Koyama 1963) and should not contribute to glow variations in *Celerio* and *Deilephila*

II Pigment migration and threshold changes in dark-adapted eyes kept in darkness

Exner (1891) suggested that in the compound eye the light attenuation varies with the pigment position According to this hypothesis the outward pigment movement causes a gradual increase in the light transmission to the retinular rhabdomeres The outward pigment migration occurring during dark adaptation in the compound eye of moths is associated with a fall in (transpigment) threshold of 2 to 3 log units (Bernhard and Ottoson 1960 a 1960 b 1964, Bernhard Hoglund and Ottoson 1963, Post and Goldsmith 1965) The relation to the pigment movement suggests that the threshold decrease is caused by a decreased screening effect of the migrating pigment This assumption is supported by the observation that the second phase of the threshold decrease is absent if the pigment fails to migrate (Bernhard and Ottoson 1960 b), and also by the results of behavioral experiments on the moth *Ephestia* (Klingebeil 1938) and electrophysiological experiments on *Calliphora* (Autrum 1955) showing that eyes lacking dark pigment are more light sensitive than normal eyes

The experiments in this section investigate the effect of the pigment migration on the light transmission and on the light sensitivity of the receptors In relation to this problem Smith and Kimeldorf (1964) found, in experiments on the moths *Agrotis* and *Pseudaletia*, that after the end of an adapting light exposure the response to β radiation reached a maximal amplitude many minutes before the response to light reached its maximum They concluded that β radiation is not attenuated by the pigment, whereas the slower increase in amplitude in response to light stimulation is due to the pigment migration On the other hand Baldwin and Sutherland (1965) reported that the time course of the increase in the amplitude of the response to X ray test flashes during dark adaptation in the cockroach *Blaberus* appears to follow pigment migration

The eyes used in the experiments described in this section had been kept in darkness for several hours before the experiments were started A maximal glow in all parts of the eyes showed that the pigment was located distally when the determinations of T and t (page 11) began The eyes were kept in darkness throughout the experiments and pigment movements were determined by observations of changes in glow size and by histological sections

RESULTS

In some eyes the glow remained maximal as long as the experiments were continued indicating that the pigment remained in the extreme distal position The maximal duration of such an experiment was 4 hours Histological sections confirmed that the pigment was located distally in all parts of the eye Figure 6 illustrates that both *T* and *t* remained constant throughout the experiments The variations in threshold did not exceed 0 2 log unit which corresponds to the accuracy of the threshold measurements

In most eyes the glow gradually diminished after the experiment was started and then disappeared indicating that the pigment had migrated in the proximal direction Such an experiment is shown in Fig 7 During the first 25 min when the glow was maximal both *T* and *t* remained constant The glow then

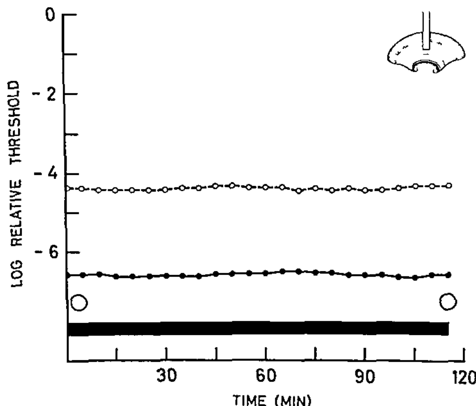


Fig 6 *T* and *t* in dark adapted eye *(Deilephila elpenor)* kept in darkness Pigment in extreme distal position Both *T* and *t* were constant throughout the experiment (For explanation of symbols see page 15)

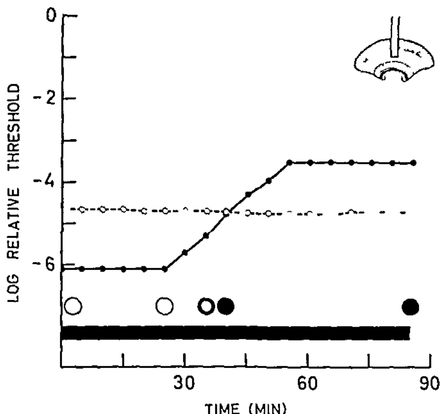


Fig 7 *T* and *t* during inward pigment migration in dark adapted eye *(Deilephila elpenor)* kept in darkness Note that pigment migration was associated with increase in *T* while *t* remained constant (For explanation of symbols see page 15)

began to diminish and simultaneously *T* began to increase The glow disappeared after 10 min The total increase in *T* was 2 6 log units, after which *T* remained constant until the experiment was terminated The glow did not reappear No glow was seen in any part of the eye at the end of the experiment indicating that the pigment had migrated inwards in all parts of the eye This was confirmed in histological sections which also showed that the fiber tip had been placed below the accessory pigment during the experiment As seen in Fig 7, *t* remained constant throughout the experiment in spite of the pigment migration

In this series of experiments the interval between the start of the experiment and the onset of the diminution of the glow varied from a few minutes in some experiments to about 2 hours in others Irrespective of the duration of this interval *T* remained constant as long as the glow was maximal and increased

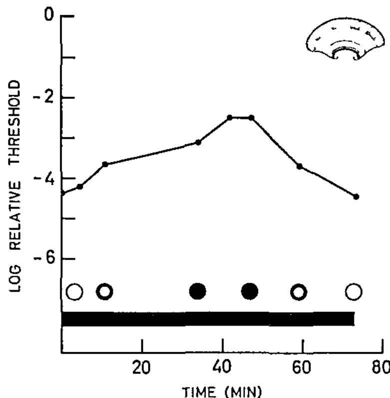


Fig 8 *T* during inward and outward pigment migration in dark adapted eye (*Noctua pronuba*) kept in darkness Note increase in *T* during inward pigment movement and decrease during outward movement (For explanation of symbols see page 15)

when the glow diminished while *t* remained constant even when the pigment migrated The maximal increase in *T* was about 2 6 log units

During the experiments the glow could be observed only within a restricted part of the eye It is well known that the pigment can migrate in some ommatidia while remaining stationary in other ommatidia (Exner 1891 Day 1941 Bernhard 1963 Yagi and Koyama 1963) Immediately after each experiment, the entire surface was therefore inspected In most experiments the glow had disappeared in all parts of the eye The only exception was found in experiments terminated shortly after the glow had disappeared in the part of the eye observed during the determinations of *T* and *t* In some of these eyes the glow could still be seen in other parts of the eye However in experiments like the one shown in Fig 7 in which the increase in *T* had reached a steady maximal value the glow had disappeared in all parts of the eye

The observation that *t* remained constant during the inward pigment move

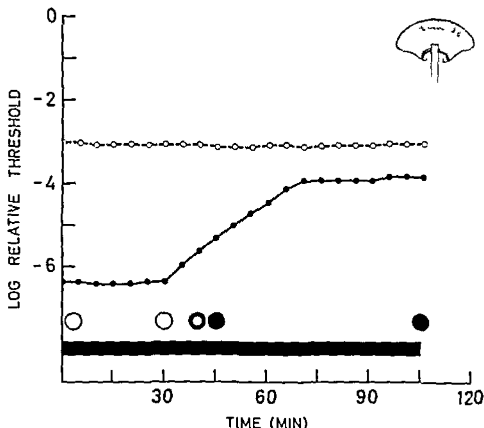


Fig 9 T and t during inward pigment migration in dark adapted eye (*Deilephila elpenor*) kept in darkness Fiber inserted through basement membrane and tip located proximal to acessory pigment (For explanation of symbols see page 15)

ment suggests that the receptor light sensitivity remained constant The in crease in T would thus be caused by an increase in light attenuation due to the pigment movement

If this interpretation is correct T would decrease in eyes where the inward pigment movement is followed by an outward movement That this is so is illustrated in Fig 8 In this experiment T increased when the glow diminished and disappeared The reappearance and increase of the glow was accompanied by a decrease in T If the retinular sensitivity remains constant when the pig ment migrates t should remain constant regardless of the position of the fiber tip between the accessory pigment and the pigment near the basement mem brane Experiments were done with the tip placed at different positions between these pigment layers The fiber was introduced near the margins as well as in the central parts of the eyes The depth to which the fiber tip was advanced

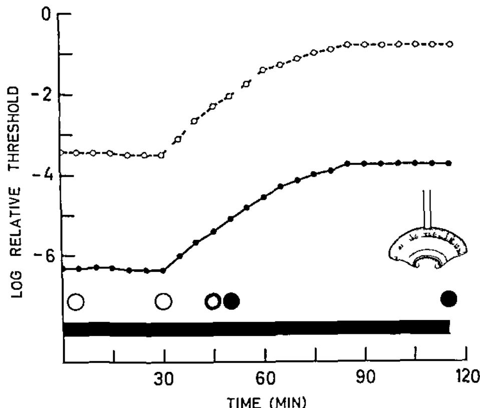


Fig 10 *T* and *t* during inward pigment migration in dark adapted eye (*Celerio euphorbiae*) *kept in darkness Fiber tip located just below inner surface of cornea Note increase* in both *T* and *t* during pigment migration (For explanation of symbols see page 15)

also varied in some eyes the tip was located near the basement membrane and in other eyes above the rhabdoms In some eyes the fiber was introduced through the basement membrane and the tip placed proximal to the accessory pigment Such an experiment is shown in Fig 9 In this experiment *t* was somewhat higher than in the experiment shown in Fig 7, which may be due to the fact that in one eye the tip pointed towards the accessory pigment (Fig 9) whereas in the other the tip pointed towards the light sensitive rhabdomeres (Fig 7) In all of these experiments *t* remained constant when the accessory pigment migrated irrespective of where the tip was located between the accessory pigment and the pigment near the basement membrane

In some experiments the fiber tip was placed just below the cornea so that the test light from the fiber passed through the accessory pigment before reaching the rhabdomeres (Fig 10) In these experiments when the pigment started

to migrate inwards, both T and t began to increase and continued to increase until they simultaneously reached their maximal values

In dark adapted eyes from which the distal pigments were removed (page 7) T and t usually remained constant as long as the experiment was continued In a few experiments both T and t slowly increased, presumably due to deterioration of the preparation In such experiments there was a simultaneous change in both thresholds

DISCUSSION

There is considerable evidence to show that the extracellularly recorded maintained cornea negative response obtained after removal or severance of the optic lobe mainly arises in the retinular cells (Bernhard 1942, Jahn and Wulff 1942, Autrum and Gallwitz 1951 Hartline, Wagner and Mac Nichol 1952 Autrum and Hoffmann 1960, Autrum Autrum and Hoffmann 1961 Goldsmith 1960, Ruck 1961) It is therefore probable that the electrical responses recorded in this study mainly originated in these cells However according to Svaetichin, Fernandez Moran and Jonasson (1956) a potential change can be recorded when an isolated corneal pigment cell preparation from moths such as *Erebus* is exposed to light The maximal amplitude of the response recorded from this preparation was 6 mV, and the authors concluded that the potential was generated in the region of the pigment cell and crystalline cone layer The question then arises whether the changes in transpigment threshold during the pigment migration were caused by a change in the threshold of an electrical response from this layer When the fiber tip was placed just below the cornea t varied with the pigment position and when the tip was placed below the accessory pigment t remained constant during pigment movements In both these experiments the light from the fiber illuminated the pigment cells It seems probable that t should have changed in both experiments if the pigment movements were accompanied by a change in electrical response from the pigment cell and crystalline cone layer sufficiently large to influence the threshold measurements It is therefore unlikely that the changes in transpigment threshold were due to variations in an electrical response from the pigment cell and crystalline cone layer

It may be asked whether the threshold changes were caused by variations in light screening by structures proximal to the accessory pigment Since movements of the pigment near the basement membrane do not exceed a few μm at the intensities used in this study it is not likely that they could alter the light screening effect sufficiently to cause a change in threshold Furthermore, if the

variations in transpigment threshold were caused by photomechanical changes proximal to the accessory pigment one would expect a change not only in the transpigment threshold but also in the infrapigment threshold It is therefore improbable that photomechanical changes in structures proximal to the accessory pigment contributed to the change in transpigment threshold

Movements not only of the accessory pigment granules but also of the pigment cell nuclei may contribute to the change in screening effect This distinction is made with reference to the observation by Edwards (1964) that movements of these nuclei may cause glow changes in eyes devoid of pigment The retinular cell nuclei do not migrate in the Sphingidae (Yagi and Koyama 1963) and therefore should not contribute to the changes in light transmission in *Celerio* and *Deilephila*

The experimental results suggest that the decrease in (transpigment) threshold associated with the outward pigment migration during dark adaptation (Bernhard and Ottoson 1960 a, 1960 b Bernhard Hoglund and Ottoson 1963 Post and Goldsmith 1965) is caused by a decrease in the light attenuation by the pigment This conclusion is supported by infrapigment threshold measurements showing that after cessation of the adapting light the photoreceptors dark adapt completely even if the pigment fails to migrate distally (page 38)

The results described here are consistent with the conclusion reached by Smith and Kimeldorf (1964) that the pigment migration during dark adaptation causes a decrease in light attenuation by the accessory pigment Baldwin and Sutherland (1965) did not specify the extent of the pigment migration in their experiments on *Blaberus* However their report that the increase in response amplitude to X ray stimulation appears to follow pigment migration does not exclude the possibility that the light transmission varies with the pigment position

The increase in light attenuation caused by the inward pigment movement may not be identical to attenuation by a neutral density filter Wave lengths in the red region of the spectrum pass through the screening pigment in *Calliphora* and *Musca* (Autrum 1955 Autrum and Burkhardt 1961 Hoffmann and Langer 1961 Burkhardt 1962 Goldsmith 1965 Langer and Hoffmann 1966) In the color blind ventral eye of *Notonecta* the spectral efficiency changes during dark adaptation possibly because red light passes through the pigment (Ludtke 1954 see also Burkhardt 1964) In flies and in *Notonecta* the screening pigment is located between the rhabdoms (see e g Goldsmith 1964 or Bullock and Horridge 1965) whereas in the moths used in the present study it is located distal to the rhabdoms Due to the structural differences between the eyes the effect of the screening pigment in moths may be different from that in flies and in *Notonecta* The observations on flies and *Notonecta* suggest however, that in

the compound eye of moths the pigment attenuates some wave lengths more than others, and that the pigment movement thereby causes a change in the spectral energy distribution of the light reaching the rhabdomeres

Pigment movements are usually induced by changes in adapting light intensity, but it is well known that movements can occur also in darkness A diurnal rhythm in the pigment position has been found in some studies on moths (Kiesel 1894 Demoll 1911, Horstmann 1935, Yagi and Koyama 1963), although in other studies no rhythmic movements were found (Collins 1934, Day 1941) It seems unlikely that the pigment movements occurring in the present experiments were due to diurnal rhythms since the pigment migrated in experiments performed during the night as well as in daytime The observation that the pigment movement took place within 2 hours, and in some eyes even within a few minutes after the experiments were started suggests that the migration should be attributed to the eyes being isolated from the rest of the body Isolation of the eye reduces the supply of oxygen and probably disturbs the normal metabolic activity of the retina In the eye of *Calliphora* the metabolic rate depends on the supply of oxygen to the retina (Langer 1962), but the electrical response from the receptor cells is very resistant to low oxygen tension (Hoffmann 1960) Earlier experimental results on Lepidoptera (Day 1941, Yagi and Koyama 1963 see Goldsmith 1964) and crustaceans (Bennit and Merrick 1932) suggest that a normal metabolic activity is required to keep the pigment in the extreme distal position

III Pigment migration and threshold changes during and after illumination of the eye

The time course of the change in (transpigment) threshold during light adaptation in the compound eye of the moth *Galleria* has recently been investigated by Post and Goldsmith (1965) They found that in most eyes the threshold changes were completed within seconds after the onset of the adapting light and then remained constant in spite of the subsequent inward migration of the accessory pigment However, in some eyes the threshold slowly increased during the pigment movement The authors concluded that the threshold increased because the increase in receptor sensitivity did not fully compensate for the increase in light attenuation when the pigment migrated proximally

A slow potential change possibly related to pigment movements has been found in the stick insect *Carausius* (Schneider 1964) In this species the amplitude of the response to (transpigment) light stimulation of constant intensity rapidly decreases during the first minute of the illumination The amplitude then remains at a constant level or in some eyes increases, for a few minutes and then slowly decreases again during about 10 to 30 min Schneider suggested that the late slow decrease in amplitude is caused by pigment migration

In the experiments to be described below T and t were determined during and after exposing the eyes to an adapting light In some experiments the adapting illumination was either weak enough or brief enough that it did not induce a pigment movement while in other experiments the intensity and duration of the adapting illumination were sufficient to induce an inward pigment movement

RESULTS

Both T and t rapidly increased when the eye was exposed to the adapting light, and at all adapting light intensities the increases in T and t were completed simultaneously in less than one minute In some experiments the difference between the increase in T and that of t was about 0 2 log unit or less In other experiments the difference was larger In such experiments t usually increased more than T The difference may to some extent be attributed to the inaccuracy of the threshold determinations However, it was mostly found when the fiber tip was placed close to the pigment near the basement membrane or close to

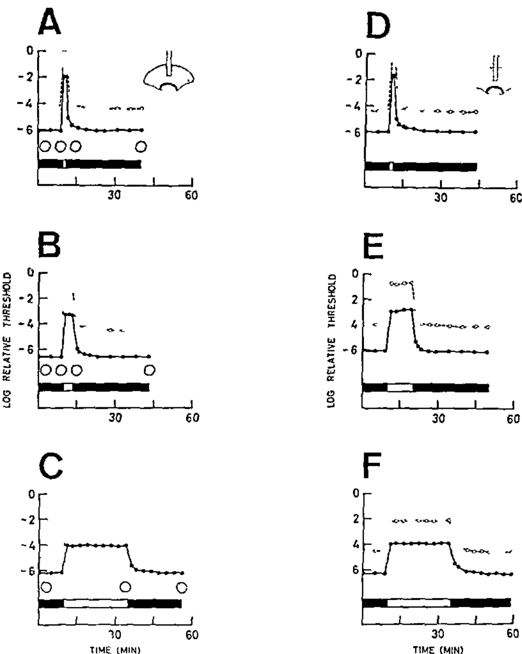


Fig 11 *T* and *t* before during and after illumination (A C D and F *Deilephila elpenor* B and E *Celerio euphorbiae*) A—C pigment in distal position during illumination D—F pigment removed Log relative adapting light intensity in A and D —1 in B and E —2 in C and F —3 Note that *T* and *t* changed almost in parallel during and after illumination Experiments made on six eyes (For explanation of symbols see page 15)

the margin of the eye These were positions at which the test light from the fiber was likely to illuminate a restricted number of ommatidia For this reason the fiber tip was usually placed centrally in the eye and usually at least 100 μm from the pigment near the basement membrane

Pigment remaining in extreme distal position or pigment removed At all intensities the glow remained maximal for some time after the onset of the adapting light Histological sections showed that as long as the glow remained maximal the pigment was situated distally in all parts of the eye In one series of experiments dark adapted eyes were exposed to light which was turned off while the glow was still maximal The changes in T and t during 3 such experiments are shown in Fig 11 A B and C Three different light intensities were used At the highest intensity (log relative intensity —1 Fig 11 A) the illumination lasted only for about 2 min since control experiments showed that with longer exposures the accessory pigment migrated proximally When log relative intensities —2 and —3 were used (Fig 11 B and C) the eyes could be illuminated for a longer period before the glow began to diminish T and t rapidly increased at the onset of the adapting light and then remained almost constant as long as the eye was illuminated When the adapting light was turned off T and t gradually decreased to the values obtained before the illumination The time course of the threshold change found in these experiments was similar to that found for the bee (Goldsmith 1963) in which no pigment movements occur

Experiments were also made on eyes from which the distal pigments had been removed Figure 11 D E and F shows that the time course of the changes in T and t during and after illumination closely followed that of the intact eye (Fig 11 A B and C) It should be added that T and t remained almost constant even when the duration of the adapting light exposure was so long that in the intact eye the accessory pigment would have migrated proximally However after the eyes had been exposed to an adapting light of high intensity for many minutes (e g log relative intensity —1 for more than 10 min) T and t decreased but to values higher than those found before the illumination which suggests that the preparation had deteriorated during the adapting light exposure

Pigment migrating during the illumination Figure 12 shows the changes in T and t when the adapting light induced an inward migration of the accessory pigment The glass fiber tip was situated close to the distal part of the rhabdoms i e well below the proximal border of the accessory pigment even when the pigment was in its most proximal position The eye was kept in darkness at the beginning of the experiment and a maximal glow indicated that the pigment was situated distally When the eye was illuminated (log relative intensity —1) T and t increased The increase was completed about 0 5 min

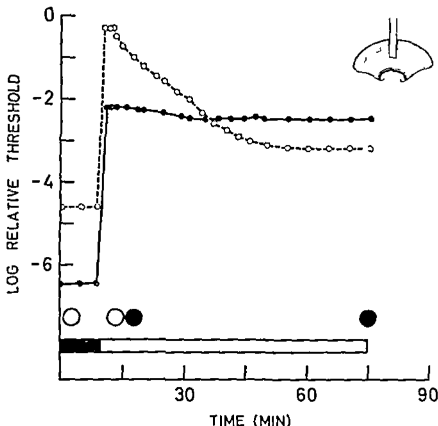


Fig 12 T and t during illumination (*Deilephila elpenor*) Pigment migrated in proximal direction during illumination Note that pigment movement was associated with decrease in t while T remained almost constant (For explanation of symbols see page 15)

after the onset of the adapting light after which T and t were almost constant The glow began to diminish 3 min after the onset of the adapting light indicating that the accessory pigment was migrating proximally At the same time t began to decrease The glow disappeared 8 min after the onset of illumination when t had decreased by 0 7 log unit The decrease in t continued until the eye had been illuminated for 45 min after which t was constant as long as the eye remained illuminated The experiment was terminated when the eye had been exposed to the adapting light for 65 min The total decrease in t was 2 9 log units In contrast to the considerable decrease in t Fig 12 shows that T decreased only 0 3 log unit

In all experiments t was almost constant during the illumination as long as the glow was maximal and decreased when the glow diminished The change in T was small compared to that of t Histological sections made after the

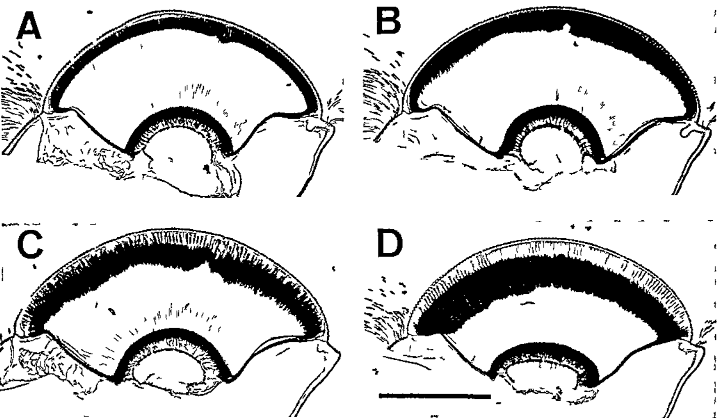


Fig 13 Histological sections of eyes (*Celerio euphorbiae*) showing pigment position after decrease in *t* during illumination Threshold decrease in A 0 log unit in B 1 1 log unit in C 2 1 log units in D 3 0 log units Horizontal bar 1 mm

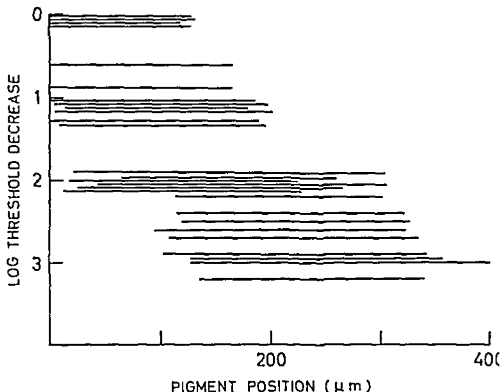


Fig 14 Relation between pigment position and decrease in t during illumination (*Celerio euphorbiae*) Values from 27 experiments Ordinate decrease in t Abscissa position of accessory pigment in μm from inner corneal surface Each horizontal line shows location in one eye Left end of bar shows position of distal pigment border and right end of bar shows position of proximal border

decrease in t was completed showed that the accessory pigment was situated proximally in all parts of the eyes

The decrease in t was correlated with the pigment position in histological sections Figure 13 A is from an experiment which was terminated before the decrease in t had begun and while the glow was still maximal The accessory pigment is situated in the extreme distal position Figure 13 B shows the pigment position in an eye when t had decreased 1 1 log unit the accessory pigment is situated more proximally The sections shown in Figs 13 C and D are from eyes used in experiments terminated when t had decreased 2 1 and 3 0 log units respectively In these eyes the accessory pigment extends still further proximally Figure 14 illustrates the relation between the change in t and the proximal migration of the accessory pigment in 27 experiments The horizontal lines represent the location of the accessory pigment expressed in μm from

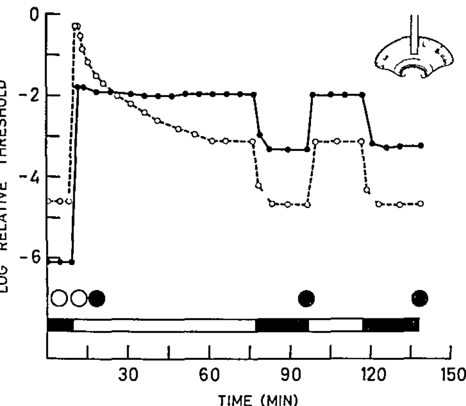


Fig 15 T and t before during and after illumination *(Deilephila elpenor)* T remained almost constant during pigment migration in first illumination period (For explanation of symbols see page 15)

the inner surface of the cornea The uppermost lines show the position of the pigment in eyes before t had started to decrease It is seen that the gradual expansion and proximal migration of the pigment was accompanied by a gradual decrease in t

The relationship between the decrease in the infrapigment threshold and the pigment movement is interpreted as follows As the pigment migrates proximally the intensity of the light reaching the rhabdomeres is gradually decreased even though the adapting light intensity is constant The decrease in the infrapigment threshold therefore represents a gradual adaptation of the receptors to the decreasing stimulus intensity and because of this increase in receptor sensitivity the transpigment threshold remains almost constant in spite of the pigment migration

Further experiments have provided additional support for this interpretation In the experiment shown in Fig 15 t decreased about 2 8 log units while T

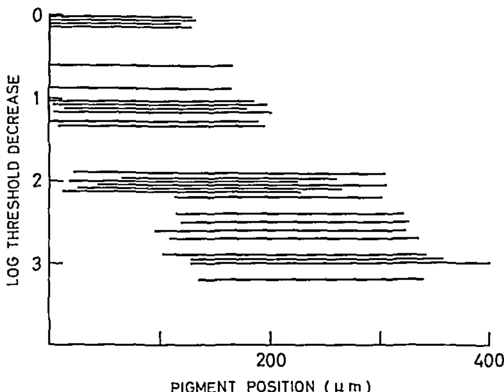


Fig 14 Relation between pigment position and decrease in *t* during illumination (*Celerio euphorbiae*) Values from 27 experiments Ordinate decrease in *t* Abscissa position of accessory pigment in µm from inner corneal surface Each horizontal line shows location in one eye Left end of bar shows position of distal pigment border and right end of bar shows position of proximal border

decrease in *t* was completed, showed that the accessory pigment was situated proximally in all parts of the eyes

The decrease in *t* was correlated with the pigment position in histological sections Figure 13 A is from an experiment which was terminated before the decrease in *t* had begun, and while the glow was still maximal The accessory pigment is situated in the extreme distal position Figure 13 B shows the pigment position in an eye when *t* had decreased 1 1 log unit, the accessory pigment is situated more proximally The sections shown in Figs 13 C and D are from eyes used in experiments terminated when *t* had decreased 2 1 and 3 0 log units respectively In these eyes the accessory pigment extends still further proximally Figure 14 illustrates the relation between the change in *t* and the proximal migration of the accessory pigment in 27 experiments The horizontal lines represent the location of the accessory pigment expressed in µm from

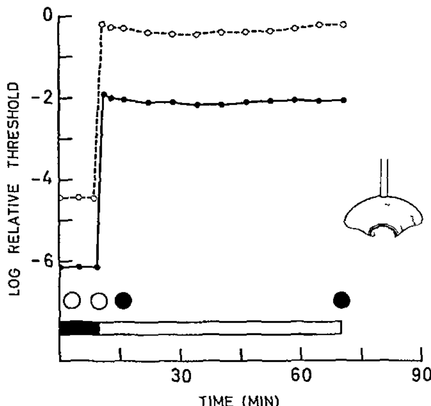


Fig 17 Fiber tip placed just below cornea whereby t was measured by light passing through pigment layer *(Celerio euphorbiae)* Note absence of decrease in t during inward pigment movement in illuminated eye (For explanation of symbols see page 15)

pigment migration The maximal increase in light attenuation was about 3 log units in this series of experiments In the experiment shown in Fig 15 the pigment movement caused an increase in light attenuation of about 2 8 log units which was compensated for by the increase in receptor sensitivity so that T remained almost constant during the pigment migration

One may predict from the preceeding experiments that the increase in receptor sensitivity represented by the decrease in the infrapigment threshold should be related to the increase in light attenuation by the pigment at any time during the adapting illumination That this is the case was shown in experiments in which the adapting light was turned off while t was still decreasing In the experiment shown in Fig 16 the adapting light was turned off at two different times while t was still decreasing The decrease in t was completed during the third light period In each dark period following these three light periods the final value for T was higher than during the preceeding dark period T was

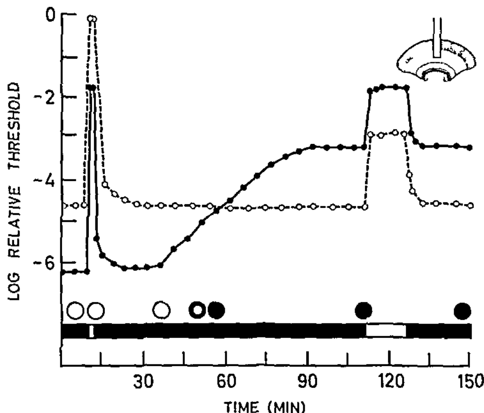


Fig 18 *T* and *t* when pigment migrated in darkness between illumination periods *(Celerio euphorbiae)* During second illumination period *t* lower than during first illumination period *T* almost equal during both illumination periods (For explanation of symbols see page 15)

the same before and after the fourth light period during which there was no decrease in *t* Histological sections of the eye showed that the pigment was located proximally at the end of the experiment

It might also be expected that if the decrease in *t* when the pigment migrates during the illumination represents an increase in receptor sensitivity, *t* should decrease only when the fiber tip is placed below the pigment Figure 17 shows that *t* did not decrease when the fiber tip was placed just below the cornea When the tip was in this position the test light passed through the pigment before it reached the rhabdomeres, and therefore both *t* and *T* were trans pigment thresholds

Pigment migrating during the dark interval between the illumination periods Figures 15 and 16 show that *T* rapidly decreased during the dark periods and then remained constant as long as the eye was kept in darkness The diagrams

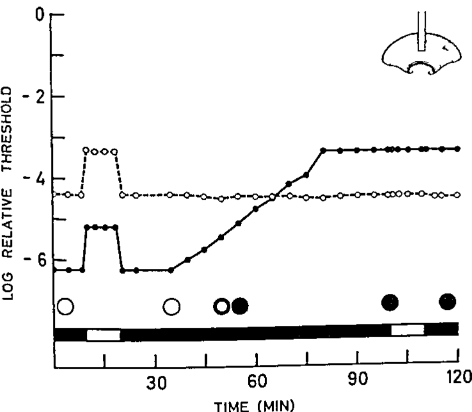


Fig 19 T and t when pigment migrated in darkness between illumination periods *(Deilephila elpenor)* During second illumination period t same as in dark adapted eye T higher during second compared to first illumination period (For explanation of symbols see page 15

also show that at the beginning of each illumination period t increased to the same value as that obtained at the end of the preceeding illumination period It can therefore be concluded that in these as in most other experiments, the proximal pigment migration stopped when the adapting light was turned off and that the pigment remained stationary as long as the eye was in darkness

In some eyes however the pigment migrated during the dark interval between two illumination periods In the experiment shown in Fig 18 the adapting light was turned off while the accessory pigment was still in the extreme distal position as evidenced by a maximal glow Since the pigment had not migrated inwards both T and t decreased to the same values as before the illumination The glow remained maximal for 25 min after which it began to diminish signalling that the accessory pigment was migrating proximally During the pigment migration T increased about 2 9 log units and then remained

constant When the eye was again exposed to the adapting light t increased to a value that was about 2 8 log units lower than during the preceeding il lumination period This experiment shows that the infrapigment threshold de pends on the screening effect by the pigment irrespective of whether the pig ment migrates during illumination or in darkness

If the light attenuation by the pigment increases to such an extent that the infrapigment threshold in the illuminated eye is about the same as that of the dark adapted eye it is to expected that the value of the transpigment thresh old also remains the same as in the dark adapted eye This transpigment thresh- old value may be higher than that observed in the illuminated eye with the pigment in the distal position Such an experiment is shown in Fig 19 The pig ment was located distally at the beginning of the experiment When the eye was exposed to the adapting light (log relative intensity —4) T and t increased about 1 1 log unit The glow remained maximal during the illumination Dur ing the ensuing dark period T and t decreased to the same values as before the illumination After the eye had been kept in darkness for 16 min the glow diminished, indicating that the pigment migrated proximally, and T increased about 2 9 log units When the eye was again exposed to the adapting light there was no change in T and t

Variations in transpigment threshold during pigment migration in the illu minated eye In some experiments the pigment movement during the illumina tion was associated with a decrease in T In one example (Fig 20) T decreased 0 6 log unit when the eye was exposed to the adapting light (log relative inten sity —1) During the first illumination period t decreased 2 9 log units When the adapting light was turned off T reached a value that was 2 2 log units higher than before the illumination

The maximal decrease in T when the pigment migrated during illumination was 0 7 log unit No increase in T exceeding 0 1 log unit was found The de crease in T which began simultaneously with the diminution of the glow was too large to be attributed to the inaccuracy of the threshold determinations *Furthermore T did not decrease during the second illumination period when* the pigment was already located proximally at the beginning of the illumina tion These observations suggest that the decrease in T was related to the in ward pigment movement Experiments were done to test if the decrease in T was caused by the adapting light being attenuated more than the test light Such a difference in attenuation could possibly have occurred since the optical path for the adapting light was not completely identical with that of the transpigment test light The optical paths for the adapting and test lights were reversed for about one minute before and after the decrease in T It was then found that the values for T were almost the same irrespective of which of the two optical paths

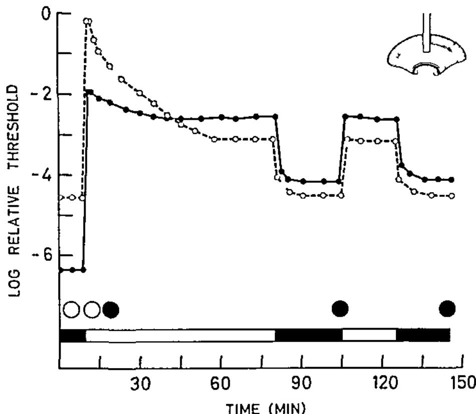


Fig 20 T and t before during and after illumination *(Deilephila elpenor)* T decreased during first illumination period (For explanation of symbols see page 15)

was used for testing T It is therefore unlikely that the decrease in T was caused by an unequal attenuation of the test and adapting lights

In most of the experiments in which T decreased during the illumination as in the experiment shown in Fig 20 the relative change in the infrapigment threshold during the illumination exceeded the difference in the transpigment threshold before and after the illumination This result indicates that in some eyes the increase in receptor sensitivity overcompensates for the increase in light attenuation by the pigment Such an overcompensation may be one reason why in some experiments T decreased during the illumination

Neutral density filters substituting for pigment screening effect In a series of experiments the relation between threshold change and change in adapting light intensity was determined This increment threshold function was obtained by determining T and t at various adapting light intensities The experiments were made on intact eyes in which the pigment remained distally as indicated

by a maximal glow, and also on eyes from which the distal pigments and cornea had been removed There was thus no change in light screening by the pigment during the experiments In both *Celerio* and *Deilephila* there was an approximately linear relationship between the change in log relative adapting light intensity and the change in log *T* and *t* from log relative adapting light intensity —1 to about —4 In experiments on 9 intact eyes from *Deilephila* and on 8 eyes of the same species from which the distal pigments and the cornea had been removed a change in log relative adapting light intensity from —1 to —4, i e 3 0 log units caused a change in *T* and *t* ranging from 3 0 to 3 6 log units The average change *in both the intact eyes and those operated on* was *3 2 log* units Thus a 1 0 log unit change in the adapting light intensity caused a change in *T* and *t* of 1 0 to 1 2 log unit (mean 1 07 log unit) This result supports the assumption that the increase in receptor sensitivity compensates and in some eyes possibly contributes to a slight overcompensation for the increase in light screening when the pigment migrates proximally

Figure 21 shows an experiment on an eye from which the distal pigments were removed together with the cornea A filter substituted for the pigment in that it attenuated the adapting light and the transpigment test light but not the infrapigment test light At the beginning of the experiment no filter was used The eye was first kept in darkness, and was then exposed to the adapting light (log relative intensity —1) The increase in *T* and *t* was completed within the first minute of the illumination after which *T* and *t* were almost constant When the eye had been exposed to the adapting light for about 5 min a filter attenuating the light by 3 log units was placed in front of the eye Upon insertion of the filter *t* decreased about 3 log units while *T* first increased slightly and then gradually decreased to about the same value as before the insertion of the filter The adaptation of the receptors to the lower light intensity was not completed until after 10 to 15 min During the early part of this period the receptor sensitivity had not increased sufficiently to compensate for the light attenuation by the filter which caused the initial increase in *T* No similar increase in *T was seen in the experiments on intact eyes (see Figs 12 15 16 and* 20) presumably because the pigment migration in the intact eye was sufficiently slow for the increase in receptor sensitivity to compensate for the increase in light attenuation at any time during the experiment

In the experiment shown in Fig 21, when the light was turned off *t* decreased to the same value as that found before the illumination The receptors thus dark adapted completely *T* decreased to a value that was about 3 log units higher than before the light adaptation since the test light was attenuated by the filter When the filter was removed *T* decreased about 3 log units When the eye was exposed a second time to the adapting light and then again kept in dark

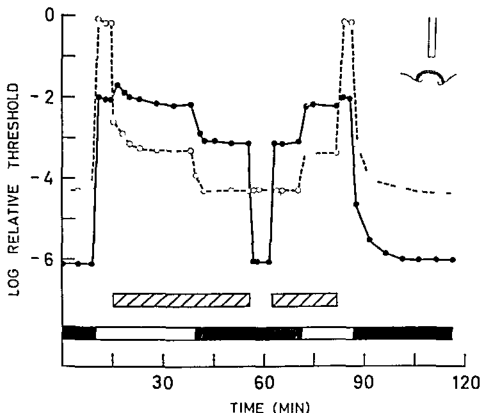


Fig 21 *T* and *t* before during and after illumination of eye from which distal pigments had been removed *(Deilephila elpenor)* Neutral density filter substituted for pigment migration (For explanation of symbols see page 15)

ness *T* and *t* reached approximately the same values as those found earlier in the experiment The experiment showed that the threshold changes observed during the pigment movements in intact eyes could be approximately reproduced when filters were substituted for the variations in light screening by the pigment It should be noted however that the increase in light attenuation caused by the pigment migration may not be identical to that caused by neutral density filters (page 29) Therefore such filters probably do not completely reproduce the changes in the stimulating light caused by the pigment movements

DISCUSSION

Little is known about the relation between pigment migration and receptor sensitivity during light adaptation Results of investigations on frogs (Dodt

and Jessen 1961 Back Donner and Reuter 1965) suggest that the pigment migration from the dark adapted to the light adapted position is associated with an increase in the sensitivity of the rods (see also Donner and Reuter 1965) The relation between the screening effect and the receptor sensitivity during light adaptation in the compound eye was recently studied by Post and Goldsmith (1965), but except for a brief previous report (Hoglund 1963 b) no direct observations of the changes in light attenuation and their effect on the receptor sensitivity seem to have been reported

The present experiments show that the screening effect of the pigment, and the receptor sensitivity, increase when the pigment migrates in the proximal direction during the adapting illumination The observations strongly suggest that the increase in receptor sensitivity compensates for the increase in light screening so that the transpigment threshold changes little during the pigment movement The same conclusion was also reached by Post and Goldsmith (1965) from their experiments on *Galleria*

By the technique used in the present study variations in the receptor light sensitivity could be directly determined during pigment movements In the assessment of results obtained by infrapigment light stimulation it should be considered that the intensity of the light illuminating retinulae close to the fiber tip is higher than the intensity of the light illuminating retinulae further away from the fiber The angle of incidence on the rhabdomeres also varies with the distance from the fiber tip Furthermore the intensity, angle of incidence on the rhabdomeres and the spectral energy distribution of the infrapigment light stimulus may be different from corresponding parameters of the transpigment light stimulus These differences probably did not significantly influence the threshold measurements because control experiments (page 44) showed that a change in adapting light intensity caused about the same relative change in the infrapigment threshold as in the transpigment threshold It is possible however that in some experiments depending on the position of the fiber tip the group of receptors that gave rise to the main part of the response to light from the fiber may not have been identical with the group of receptors that gave rise to the transpigment threshold response If in such an experiment the pigment migrated more in some ommatidia than others the observed change in transpigment threshold may not have been a correct measure of the change in light screening that caused the observed change in the infrapigment threshold Furthermore the pigment migration may cause changes in the light transmitted through the pigment that do not affect the infrapigment test light (page 29) For these reasons no definite conclusions have been drawn from a comparison between the numerical values for the relative changes in the infrapigment and transpigment thresholds

The assumption that in some eyes the increase in receptor sensitivity over compensates for the increase in screening effect by the pigment (page 43) is partially based on a comparison between the numerical values for the relative changes in the infrapigment and transpigment thresholds Even though support for this assumption was obtained from measurements of the infrapigment and transpigment thresholds at various adapting light intensities (page 44) the experimental results are only regarded to indicate that such an overcompensation sometimes occurs Similar findings were obtained in *Galleria* by Post and Goldsmith (1965) They found that in some eyes the inward pigment migration during illumination was accompanied by an increase in (transpigment) threshold and that a change of one log unit in adapting light intensity caused a smaller change in threshold In the present study the change in log relative threshold sometimes exceeded the change in log relative adapting light intensity and the transpigment threshold decreased during the movement

The report that an electrical potential change can be recorded when the isolated corneal pigment cell preparation from *Erebus* is exposed to light (Svaetichin Fernandez Moran and Jonasson 1956) raises the question whether in the present study part of the decrease in infrapigment threshold was caused by a decrease in the light intensity necessary to elicit a response from the pigment cells It is unlikely however that more than a small part of the decrease in the infrapigment threshold reported here was caused by such a change in pigment cell threshold as evidenced by the results of experiments made on eyes from which the cornea and a large part of the pigment cells were removed (page 44) In these eyes as in intact eyes a change in light attenuation of one log unit caused a threshold change of one or slightly more than one log unit which suggests that the greater part of the decrease in the infrapigment threshold in intact eyes was caused by the increase in receptor sensitivity It cannot be excluded however that a decrease in threshold of an electrical response from the pigment cells in intact eyes contributed to the threshold decrease by a few tenths of a log unit

Schneider (1964) in his experiments on *Carausius* measured the amplitude of the response to (transpigment) test flashes of constant intensity He suggested that the slow decrease in amplitude observed during illumination of a previously dark adapted eye may be due to positional changes of the screening pigment It is possible that this slow decrease corresponds to the slow changes in threshold sometimes found during the inward pigment movement when moth eyes are exposed to light In locusts the initial increase in (transpigment) threshold when the eye is exposed to an adapting light is followed by a slow decrease (Burtt Catton and Cosens 1964) The threshold changes in the locust eye were determined by measuring the test light intensity needed to evoke a spike dis

charge in the nervous system Burtt, Catton and Cosens (1964) found that the time course of these changes resembles the time course of a DC shift recorded in the retinular cell zone (Burtt and Catton 1964) These variations in DC potential are abolished after removal of the optic lobe (Burtt and Catton 1964) which indicates that the slow threshold decrease is not caused by pigment movements

The experiments described in the present paper have investigated the relation between the receptor sensitivity and the screening effect by the pigment It should be noted that the receptor sensitivity may also be affected by other factors such as the activity of the optic lobe and perhaps also by endocrine organs In the water beetle *Dytiscus* the threshold of the dark adapted eye to (transpigment) light stimuli is about a thousand times higher during the day than during the night and the difference in sensitivity is unrelated to variations in pigment position (Jahn and Wulff 1941, 1943) Furthermore, activity in the optic lobe has been reported to influence the electrical response to light stimulation in several other insect species e g in flies (Autrum 1958 Burtt and Catton 1964) in locusts (Burtt and Catton 1964), and in the butterfly *Heliconius* (Swihart 1964)

Kuiper (1962) has suggested that the pigment protects the receptor cells from overillumination The observation that the receptor sensitivity increases during the inward pigment migration, and thereby compensates for the decreasing intensity of the light reaching the rhabdomeres suggests in accordance with Kuiper's hypothesis, that one function of the migrating pigment is to reduce the intensity of the receptor stimulation with little loss in the sensitivity to (transpigment) light stimuli

In moths the changes in sensitivity to (transpigment) light stimuli are usually completed rapidly after a sudden change in the adapting light intensity, e g when a shadow is cast on the eye even though the pigment migration is slow However the change in sensitivity is rapidly completed only if the change in adapting light intensity is moderate If a previously brightly illuminated eye *with the pigment in the proximal position is suddenly exposed to a very low* adapting light intensity the pigment remains for some time in the proximal position Until the pigment has migrated to a more distal position the light may be attenuated to such an extent that no response is evoked in the retinular cells even if they have gained the same sensitivity as in a completely dark adapted eye The intensity of the light reaching the rhabdomeres may also be too low to excite the retinular cells during pigment movements that are not induced by changes in the adapting light intensity such as rhythmic pigment movements When the change in the adapting light intensity is slow, e g during sunrise and sunset the pigment movements follow the change in the light intensity so that

the intensity of the light reaching the rhabdomeres changes less than the adapting light intensity.

It is of interest that in the frog the screening effect by the pigment, and thereby the rhodopsin content in the illuminated eye, varies with the pigment position (Back, Donner and Reuter 1965). This finding, together with the report that the pigment position affects the threshold of the illuminated eye (Dodt and Jessen 1961), suggests that the relation between pigment light screening and receptor sensitivity may be partially similar in arthropod and vertebrate eyes.

SUMMARY

1. The relation between the photoreceptor light sensitivity and the positional changes of the retinal screening pigment was studied in the compound eye of the moths *Celerio euphorbiae* and *Deilephila elpenor* (Sphingidae).

2. Relative thresholds were measured electrophysiologically in darkness and during illumination by *a* test light passing through the cornea and distal pigments (the transpigment threshold) and *b* test light conducted through a glass fiber, the tip of which was placed below the accessory pigment (the infrapigment threshold).

3. The pigment position in histological sections of eyes exhibiting maximal or minimal glow was determined. It was found that the proximal and distal borders of the accessory pigment were located more proximally in eyes with minimal glow than in eyes with maximal glow. Changes in glow size between maximal and minimal were therefore used as index of pigment movements. No movements of pigment near the basement membrane exceeding a few μm were found.

4. The threshold measurements showed that in dark adapted eyes the infrapigment and transpigment thresholds were constant as long as the pigment remained in the extreme distal position. An inward pigment migration in dark adapted eyes kept in darkness was associated with an increase in the transpigment threshold while the infrapigment threshold remained constant. An outward pigment migration was associated with a decrease in the transpigment threshold.

5. The infrapigment and transpigment thresholds reached a steady value in less than one minute after the onset of adapting light. The thresholds then remained almost constant as long as the pigment remained in the extreme distal position. Cessation of the adapting light with the pigment in the extreme distal position was followed by a gradual decrease in the infrapigment and the transpigment thresholds to the same values as those found before the illumination. Almost the same time course of threshold change was found in eyes from which the distal pigments had been removed.

6. The inward pigment migration during exposure to adapting light was associated with a decrease in infrapigment threshold. The transpigment threshold remained constant or decreased slightly during the pigment migration. Upon

cessation of the adapting light the infrapigment threshold decreased to the same value as that found before the illumination although the pigment did not migrate distally during the dark period The transpigment threshold decreased to a value that was higher than before the illumination

Upon re exposure of the eye to the adapting light the infrapigment threshold increased to about the same value as that observed at the end of the preceeding illumination, provided the pigment did not migrate during the intervening dark period When there was an inward pigment migration during the intervening dark period the infrapigment threshold upon re illumination increased to a value lower than at the end of the preceeding illumination The transpigment threshold increased to about the same value as that observed at the end of the preceeding illumination irrespective whether the pigment migrated during the intervening dark period However if the infrapigment threshold remained the same in the illuminated as in the dark adapted eye the value of the transpigment threshold upon re illumination was higher than the value observed at the end of the preceeding illumination

7 In most experiments in which the transpigment threshold decreased during the pigment migration occurring during exposure to adapting light the relative change in infrapigment threshold during the pigment movement exceeded the difference between the transpigment threshold before and after illumination Increment threshold experiments showed that a change in the adapting light intensity of 1 0 log unit caused the infrapigment and transpigment thresholds to change 1 0 to 1 2 log unit The threshold changes observed during the pigment migration could be approximately reproduced when neutral density filters were substituted for the variations in light screening by the pigment

8 The experimental results show that in dark adapted eyes kept in darkness pigment movements may occur with no change in the receptor light sensitivity The light attenuation varies with the pigment position and thus causes variations in the sensitivity to (transpigment) light stimuli

During illumination the pigment migrates proximally Due to the increase in light attenuation the intensity of the light reaching the rhabdomeres gradually decreases The photoreceptors adapt to the decreasing stimulus intensity with *an increase in their sensitivity The sensitivity to (transpigment) light stimuli changes only slightly during the pigment movement because the increase in* receptor sensitivity usually compensates for the increase in light attenuation The increase in receptor sensitivity may in some eyes contribute to an over compensation for the increase in light attenuation The increase in light attenuation is not compensated for if the inward pigment migration continues after the receptors have attained the same sensitivity as in the dark adapted eye

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ESTERASES OF THE RAT ADENOHYPOPHYSIS

CELLULAR LOCALIZATION AND ACTIVITY IN RELATION TO SECRETORY FUNCTIONS

BY

TAPANI VANHAPERTTULA

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PREFACE

The present study was carried out at the Department of Anatomy, University of Turku in 1963—1966.

I wish to express my sincere thanks to Assistant Professor Väinö K. Hopsu, M.D., who suggested me this field of study and who gave me encouragement and valuable advise throughout the investigation. I am also grateful to Professor Mikko Niemi, M.D., the present Head of the Department for his critical and valuable suggestions in the preparation of the manuscript.

My best thanks are due to my colleagues at the Department for pleasant collaboration and friendly dealings. I wish warmly to thank the technical personnel of the Department, especially Mrs. Marja Oksala for her skilful technical assistance and Mr. Paavo Viljanen for the great trouble he took with the photographs.

I am further indebted to Dr. William Waddell, M.D. from the Department of Pharmacology, University of North Carolina, Chapel Hill, USA for checking the language of the manuscript and his interest in my work. I am grateful to Mrs. Aili Ryynanen, M.A. and her assistents at the Library of the Medical Faculty for their help in obtaining literature. My thanks are also due to Mr. Heikki Lopponen, M.A. for his advise in statistical problems and to Mr. Unto Alapiessa, M.Sc. for the generous supply of the steroid hormones of N.V. Organon used in the present work.

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Turku, September 1966

Tapani Vanha-Perttula

CONTENTS

INTRODUCTION

Several different types of parenchymal cells can be demonstrated in the rat anterior pituitary gland by a number of histological and histochemical techniques However these techniques do not specifically demonstrate the pituitary hormones themselves Therefore an experimental approach has frequently been used in order to localize the site of synthesis and/or storage of a specific hormone to a certain cell type This usually includes observations on the pituitary cytology after total extirpation of different endocrine glands or after experimentally induced alterations in their functional status During recent years a more direct approach has become available for the cellular localization of some pituitary hormones and this is based on the use of fluorescent labelled antibodies to these hormones

Any synthesis and release of hormones must obviously rely on a series of reactions coupled with a number of more or less specifically acting enzyme systems Unfortunately only a limited number of such enzymatic activities can be localized histochemically to a specific cell Thus most of the enzymes active in protein and polysaccharide synthesis and consequently connected with the synthesis of the anterior pituitary hormones cannot be demonstrated at the cellular level On the other hand a number of histochemically demonstrable enzyme activities have been found specifically distributed among the pituitary cells The enzymes with an esterolytic capacity are especially active in the anterior pituitary gland but the role of this class of enzymes for hormone production has not been worked out Only a few published reports deal with changes in histochemically demonstrable esterase activity in experimentally altered pituitary function Quantitative measurements of the total esterase activity of the pituitary gland under experimental conditions are completely lacking

Observations leading to the discovery of specific enzymes taking part in the hormone production or release may be expected to aid in the distinction of the pituitary cell types However pure biochemical characterization of a specific enzyme of the pituitary gland is not suitable for

correlation of enzymic activity to the production of a certain hormone because of the multiple cellular composition of the gland. Microscopic histochemical investigation should be therefore combined with biochemical assays to explore the specific metabolic characteristics of the pituitary cells.

Histochemically demonstrable esterases give an example of a heterogeneous group of enzymes, whose biochemical characteristics can not be adequately described using conventional histochemical investigation. Biochemical separation of a tissue homogenate and further characterization of the partially purified esterase fractions using different substrates and modifier agents are necessary to complete an investigation of the esterases in a tissue like the pituitary gland. Quantitative determination of the esterase activity in different functional states of an endocrine gland is obviously of importance for an evaluation of the significance of the enzyme to hormone production.

HISTOLOGY OF THE RAT ADENOHYPOPHYSIS

LITERATURE REVIEW

Parenchymal cells

The anterior pituitary gland secretes the following *protein hormones* somatotropic or growth hormone (STH), prolactin or lactotropic hormone (LTH) corticotropic hormone (ACTH) and melanotropic or melanocyte stimulating hormone (MSH) in man (Morris et al 1956 Purves 1961 Hermanus et al 1964) and in species which have no intermediate lobe (Tixier Vidal 1963) The *glycoprotein hormones* produced by the pituitary gland are follicle stimulating hormone (FSH) luteinizing hormone (LH) or interstitial cell stimulating hormone (ICSH) and thyrotropic hormone (TSH) [The nomenclature follows the recommendation of the International Committee for the Nomenclature of the Adenohypophysis (van Oordt 1965)]

The localization of these hormones in the different cell types in the anterior pituitary gland has been an intriguing problem for many decades From the abundant studies concerning the pituitary morphology in the rat only the most important contributions are presented schematically in Table I

Table I Summary of the literature ascribing the production of different hormones to specific types in the rat pituitary gland included are the immunofluorescent studies in different spe

Hormone	Staining (Mallory)	Cell type (Herlant)	Special feature	Reference
STH	acidophil	α	orangeophile	Dawson 1954
			carminophile	Sanders & Rennels 1959
			phospholipid positive	Herlant 1959 Rennels 195
			orangeophile (tetrachrome and Cleveland Wolfe staining)	Pasteel & Herlant 1962
			degranulation after thyroidectomy	Knigge 1958
			immunofluorescence (man)	Leznoff et al 1960
				Grumbach 196
				Pearse & van Noorden 196
			immunofluorescence (ox, sheep rat, mouse)	Rumke & Ladiges 1965

correlation of enzymic activity to the production of a certain hormone because of the multiple cellular composition of the gland Microscopic histochemical investigation should be therefore combined with biochemical assays to explore the specific metabolic characteristics of the pituitary cells

Histochemically demonstrable esterases give an example of a heterogeneous group of enzymes, whose biochemical characteristics can not be adequately described using conventional histochemical investigation Biochemical separation of a tissue homogenate and further characterization of the partially purified esterase fractions using different substrates and modifier agents are necessary to complete an investigation of the esterases in a tissue like the pituitary gland Quantitative determination of the esterase activity in different functional states of an endocrine gland is obviously of importance for an evaluation of the significance of the enzyme to hormone production

ble I (continued)

rmone	Staining (Mallory)	Cell type (Herlant)	Special feature	Reference
[or 3H	basophil	γ	PAS + central, round	Purves & Griesbach 1954, 1955 1956
			PAS red peripheral	Rennels 1957, 1963 Hildebrand et al 1957
			immunofluorescence (man)	Midgley 1963 Koffler & Fogel 1964 Pobyn et al 1964
3H	basophil	β	PAS + peripheral	Purves & Griesbach 1954 1955 1956
			PAS purple, central	Rennels 1957 1963 Hildebrand et al 1957
			immunofluorescence (man)	Koffler & Fogel 1964
			immunofluorescence (pig)	Della Corte & Biondi 1964

The site of the *somatotropic hormone* production in acidophilic cells of the pituitary gland was suggested first by the observation that acidophilic adenomas produce symptoms of acromegaly and gigantism (Cushing and Davidoff 1927) These findings have been specifically confirmed by immunofluorescent studies in man (Leznoff et al 1960 Grumbach 1962 Pearse and Van Noorden 1963b) and in different animals including the rat (Rumke and Ladiges 1965) In the rat hypophy is these cells rapidly lose their granules after thyroidectomy or after the administration of antithyroid drugs and the degranulation can be prevented by the administration of thyroxine (Zeckwer et al 1935 Purves and Griesbach 1946 Halmi 1950 Griesbach 1953) The acidophilic cell secreting STH is orangeophilic according to the classification of Dawson and Friedgood (1938)

Another type of acidophilic cell in a number of animals is carminophilic and erythrosinophilic These cells are present also in the rat pituitary gland and are most active during pregnancy and lactation (Dawson 1963) The granules of these cells are depleted during the suckling of the litter which agrees with the earlier finding that suckling causes a rapid fall in the *lactotropic hormone* content of the pituitary gland (Grosvenor and Turner 1958) Based on the differential solubilities of the pituitary tropic hormones in trichloroacetic acid solutions Barrnett et al (1961) have located LTH activity in the acidophilic series of rat pituitary cells Immunologic confirmation *for the localization of LTH in acidophils has also been performed in the rat* pituitary gland (Emmart et al 1963 Rumke and Ladiges 1965) According to Sanders and Rennels (1959) LTH is secreted by the orangeophilic variant of the acidophilic cells and STH by the carminophilic cells

The studies concerning the cellular origin of the *corticotropic hormone* in different animal species have been recently summarized by Purod (1964) All the cell types in the pituitary gland have been suggested at one time or another to be the site of ACTH secretion Even the immunofluorescent investigations have given controversial results (Marshall 1951, Leznoff et al 196 Pear and Van Noorden 1963b) The cellular

ite of ACTH production in the rat has been thought to reside in acidophilic cells (Kracht et al 1955a b) Autoradiographic studies by Siperstein (1963) and Knutson (1966) as well as electron microscopic studies by Farquhar (1957), Siperstein and Allison (1965) and Kurosumi and Kobayashi (1966), on the other hand indicate chromophobes as ACTH producers A quite new site of the ACTH production in the rat was suggested by Herlant and his colleaque This was a chromophobe cell which contains a seed bed of erythrosinophilic granules at one end of the cell when stained by the tetrachrome method (Herlant 1960a) A definite hypertrophy of this cell type was found after adrenalectomy (Quenum and Herlant 1964) as well as after such drugs as amphenone and metopirone (Racadot and Herlant 1960 Herlant and Klatersky 1963, Racadot 1963a c) These cells are involuted after cortisone treatment (Racadot 1963c) The histologic appearance of this cell type in the rat anterior pituitary gland greatly resembles that of LTH cells in Herlant s tetrachrome method (Pasteels and Herlant 1962)

A different localization of *gonadotropic hormones* in the PAS positive cells was suggested by Purves and Griesbach (1954 1955) FSH seemed to be secreted by peripheral coarse granulated cells and LH by centrally situated larger, and fine-granulated cells An opposite opinion of the location of LH and FSH activities in the rat pituitary gland has been presented however (Rennels 1957 1963 Hildebrand et al 1957, Hellbaum et al. 1961) By using a PAS methyl blue orange G method some cells are stained red and are situated peripherally in the so called ' ex zone ' An increase of this cell type after gonadectomy coincides with an increase in LH activity whereas high FSH activity can be found when PAS purple central gonadotrophs were increased in number Strain differences may explain a part of this controversy (Rennels 1963)

Since the studies of Halmi (1950, 1952a b) and Purves and Griesbach (1951a, b, c, 1956) there has been a unanimous opinion of the site of the *thyrotropic hormone* production in the central basophilic cells, which have a specific affinity to aldehyde-fuchsin (Halmi 1950) and aldehyde thionin (Paget and Eccleston 1960 Ezrin and Murray 1963)

Cleft epithelial cells

The anterior pituitary gland of the rat is separated from the intermediate lobe by a cleftlike space which is covered by epithelial cell linings on both sides When the anterior lobe is separated mechanically from the intermediate and posterior lobes the anterior epithelium follows the anterior lobe tissue Therefore some properties of these cell coverings must be presented The lining cells of the cystlike cavities between anterior and posterior lobes in man secrete colloidal material into the cysts (Romeis 1940) The function of this colloid is still unknown In the rat pituitary the sickle shaped cleft between anterior and intermediate lobes remains throughout the life of the animal

The anterior and posterior epithelial linings have differences in their cellular characteristics (Ferrer 1956) The posterior epithelium lining the intermediate lobe varies from flattened cuboical, or cylindrical to stratified epithelium and different cell types can be identified Colloid droplets have been found in the apical zones of the cells and the colloid in the pituitary cleft is probably mainly the product of apocrine secretion of these cells Increased secretion has been found after castration and ad

renalectomy The anterior epithelium is low Through fenestrations in the epithelial lining basophils, acidophils and capillaries can project into direct contact with the cleft Remnants of the anterior pituitary cell transudation from the capillaries as well as mucus from the goblet cells have also been thought to contribute to the colloid formation (Ferrer 1956)

Vascular and perivascular cells

The sinusoidal capillaries lead the blood from the base of the hypothalamus to the anterior pituitary gland The studies of Cappell (1929) have shown that the pituitary capillaries are formed by endothelial cells and, in the perisinusoidal space by perivascular cells which have the property of phagocytosis (Rinehart and Farquhar 1955 Farquhar 1961) Around these sinusoids fine reticular fibres can be seen in the homogeneous ground substance These fibres as well as the ground substance are PAS positive

The perisinusoidal spaces extend between the parenchymal cells thus allowing the cells a large surface area to liberate their granules The actual liberation of secretory granules of glandular cells into this space has been verified by electron microscopic studies (Farquhar 1961, Sano 1962 Herlant 1963 Maillard 1963, Weiss 1965)

Different opinions have been presented concerning the presence of mast cells in the pituitary gland Consolandi and Briziarelli (1952) have found consistently mast cells in human material and in addition a micro form, which they call Micromastzellen In a recent communication of Garvie (1965) very few mast cells were found in the anterior pituitary gland of the rat

OWN INVESTIGATIONS

Material and methods

The material consisted of adult Long Evans rats weighing 180—260 g at the beginning of the experiment they were subjected to conditions which are known to change the physiological state and histological picture of the adenohypophysis These included male rats which were castrated adrenalectomized, injected with testosterone (1 mg/day Neo Hombreol® Organon), cortisone (1 mg/day Adreson® Organon), fed with thyroxine (0 2 % mixed in food Thyranon® Organon) or with methylthiouracil (MTU 2 % mixed in food 4(6) methyl 2 thiouracil Fluka AG) The experimental conditions of female rats were as follows castration injections of estrogen (0.1 mg/day Dimenformon®, Organon), progesterone (1 mg/day Progestin® Organon) or reserpine (0 05 mg/day Serpasil®, Ciba) pregnancy (from 12th to 21st day) lactation (from *3rd to 10th day*), *12 hours* after discontinuation of lactation and immature female rats

The experimental conditions lasted 15 30 and 60 days Each group consisted 6 rats, 2 animals were killed at each of the three time periods and their pituitary glands were removed immediately and fixed for 8 hours in modified Bouin Hollande fixative as presented by Herlant (1960a)

After deparaffinization the 5 μ sections were treated for 3 min in Lugol s solution and then in 5 % sodium thiosulphate solution in order to remove mercury salts. The

following staining methods were used periodic acid Schiff orange G (Pearse 1949 1950 1961) aldehyde fuchsin (Halmi 1952a), alcian blue at pH 3 0 and pH 0 2 (Herlant 1960a), tetrachrome method (Herlant 1960a), acid fuchsin aniline blue (Fisher and Bulmer 1964) aldehyde thionin (Ezrin and Murray 1963) and azocarmine orange G (Dawson and Friedgood 1938)

Results

Characteristics of the parenchymal cell types

The identification of the different pituitary cell types was based on the findings obtained by using the different staining techniques on successive histological sections The tentative identification was confirmed by recording specific changes in different cell types during the experimental conditions The tinctorial characteristics of the cells thus became evident and are presented in Table II In the following, different cell types are separately described The characteristics of the cells are mainly based on the staining method which gave the best differentiation

Somatotropic cell — The most abundant chromophilic cell type in the pituitary gland of the control rats and young immature rats was a small round or oval cell which was strongly orangeophilic in sections stained with methods which included orange G In the azocarmine orange G method this cell type acquired ochre or light brown coloring (Fig 1) This cell type is quite uniformly distributed throughout the whole anterior pituitary gland Sometimes these cells are found in small groups or line along the sinusoids Immature rats have an abundant number of these cells and during testosterone treatment these cells obtained an increased affinity for orange G After methylthiouracil feeding the intense staining of these cells was decreased and only some ochre stained granules were found in the cytoplasm and the cells look vesicular out These somatotropic cells were intensely red stained in the acid fuchsin aniline blue method but could not be differentiated from the lactotropic cells in pregnant and lactating female rats This differentiation could, however be achieved by the tetrachrome method in addition to the azocarmine orange G method Using the former method somatotropic cells were orange stained but in this method the concomitant aniline blue readily overstains these cells Thus the latter method is more specific for the demonstration of somatotropic cells

Lactotropic cell — Another cell type with orangeophilic properties in the PAS orange G staining method was present in augmented number during pregnancy lactation and after treatment with estrogen The azo carmine orange G method resulted in a carminophilic staining (Fig 1)

Table II Tinctorial characteristics of the different cell types after various staining methods

Cell type	PAS orange G	Aldehyde fuchsin	Alcian blue		Tetrachrome	Acid fuchsin aniline blue	Aldehyde thionin	Azocarmine orange G	Nomenclature (Herlant)	Localization
			pH 3.0	pH 0.2						
STH	orange	unstained	orange	orange	orange	red	orange	ochre	alpha	in small groups
LTH	orange	unstained	orange	pale red	brick red	red	orange	carmine	eta	separately or in palissades
ACTH	orange granules	unstained	unstained	unstained	violet granules	violet granules	orange granules	violet granules	epsilon	separately or in palissades
TSH	red	red	green	green	dark blue	blue	dark blue	dark blue	delta	central
LH	red	unstained	light green	red	light blue	violet	red	light blue	gamma	central
FSH	red	unstained	violet	violet	light blue	violet	light blue	light blue or violet	beta	peripheral
Endothelium	red	pink	red	unstained	blue	unstained	blue	blue		
Pericytes	red	pink	red	pink	blue	blue	blue	blue		

following staining methods were used: periodic acid Schiff orange G (Pearse 1949, 1950, 1961), aldehyde fuchsin (Halmi 1952a), alcian blue at pH 3.0 and pH 0.2 (Herlant 1960a), tetrachrome method (Herlant 1960a), acid fuchsin aniline blue (Fisher and Bulmer 1964), aldehyde thionin (Ezrin and Murray 1963) and azocarmine orange G (Dawson and Friedgood 1938).

Results

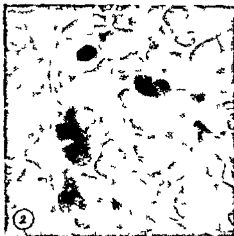
Characteristics of the parenchymal cell types

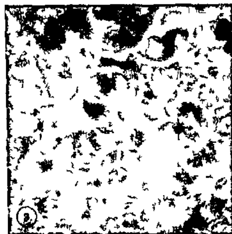
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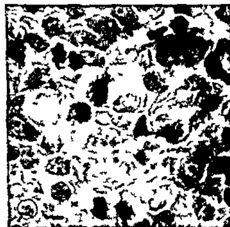
Lactotropic cell. — Another cell type with orangeophilic properties in the PAS orange G staining method was present in augmented number during pregnancy, lactation and after treatment with estrogen. The azocarmine orange G method resulted in a carminophilic staining (Fig. 1)


1


2


3


4




5

Thyrotropic cell — The identification of the thyrotropic cells by the present methods was quite easy With aldehyde fuchsin only certain central polygonal cells are stained red The same cells are dark blue when the aldehyde thionin method was used (Fig 4) Alcian blue at pH 0 2 stained these cells green, while at pH 3 0 also other cells had some though weaker affinity for this dye (Fig 6) These AF positive cells are quite large cells, contain fine granulation and are not intimately bordering the sinusoids, but come into contact with these by their branches Sometimes the cells are arranged in a grape like fashion along a sinusoid After methylthiouracil feeding, a greatly increased number of these cells was observed and also small irregular groups of these cells were present Thyroxin treatment greatly diminished the number of the TSH cells and those found were shrunken

TSH cells had a strong affinity for PAS staining using acid fuchsin aniline blue as well as tetrachrome methods, the intensity of the blue stain in these cells was stronger than in other mucoid ' cells

FSH cell — FSH cells are quite similarly stained by the present methods with the gonadotropic cells which secrete LH The methods by which differentiation can be obtained are the alcian blue method at pH 0 2 and the aldehyde thionin method In the former, FSH cells are stained violet due to the affinity of these cells for alcian blue and PAS staining while the latter component stains only LH cells In the azocarmine orange G method the FSH cells are of somewhat violet hue due to small granules (Fig 5) The distribution of FSH cells in the rat pituitary gland was characteristic They are mostly peripheral in the ventral part of the gland, but a large group of cells was present in the s c 'sex zone adjacent to the intermediate lobe FSH cells are round or oval in shape have a great affinity for PAS and are grouped around the sinusoids The granules are coarse, but leave a round Golgi zone free After castration a marked enlargement of these cells takes place and, after a longer time vacuoles appear in the cytoplasm Thirty days after castration some typical castration cells are formed with signet ring appearance In young rats at about 30 days of age a large number of FSH cells was present on the ventro caudal border of the pituitary gland

LH or ICSH cell — LH cells are quite few in the normal male and female rat but after castration and during pregnancy a large number of cells appear which have a great affinity for PAS staining At pH 0 2 these cells have only weak affinity for alcian blue and remain red due to the PAS staining At pH 3 0 these cells are, however light green colored (Fig 6) These cells are centrally situated but are easily separable from

the TSH cells by their round or oval shape and lighter staining affinity in methods like tetrachrome acid fuchsin aniline blue and azocarmine-orange G The glycoprotein granulation is finer than that of FSH-cells. These cells are also intimately connected to the sinusoids but are not arranged in larger groups

Other cell constituents of the adenohypophysis

The capillary endothelial cells are somewhat PAS positive and stain red in the PAS orange G and alcian blue pH 3 0 methods By the acid fuchsin aniline blue method these cells remain almost unstained but small fusiform cells in the perisinusoidal space are strongly blue stained These perisinusoidal cells as well as capillary endothelial cells are blue both in azocarmine orange G, tetrachrome and aldehyde thionin methods

Another cellular component which is intimately connected to the anterior pituitary tissue are the two sheets of cells lining the pituitary cleft between the anterior and intermediate lobes The posterior epithelium covers the cleft on the side of the intermediate lobe and the anterior epithelium separates the anterior pituitary cells from the cleft The latter cell line follows the anterior lobe when this is separated mechanically from the other lobes

The posterior epithelium varies in different places it is sometimes cubical columnar or even stratified especially at the angles of the anterior and intermediate lobes and in places where blood vessels pierce the intermediate lobe Differences in the height of the posterior epithelium has been recorded earlier in different experimental conditions, thus ad renalectomy and gonadectomy causes a flattening of the epithelium (Ferrer 1956) PAS positive goblet cells are also found between the epithelial cells

The anterior epithelium is not continuous Both acidophilic and basophilic cells push between the epithelial cells into a connection with the cleft lumen Increased basophilic cell penetration is found after castration and the basophil cells in question seem to be FSH gonadotrophs The colloid content in the cleft is much increased after castration but can also be found in control animals and after adrenalectomy Young animals and those receiving estrogen testosterone or cortisone are devoid of colloid When a large amount of colloid is present both the anterior and the posterior epithelial linings are decreased in height Vacuoles of different size can then be seen in colloid close to the epithelial cells The colloid in the pituitary cleft stains red with PAS staining violet red with aldehyde

thionin and blue with azocarmine orange G Within the colloid, sometimes fragments of anterior pituitary basophils or acidophils can be found together with some erythrocytes

Discussion

The simultaneous demonstration of all the different pituitary cell types by a single staining method appeared to be impossible Thus a combination of different staining techniques was used in order to reveal specifically a certain cell type The topographic distribution pattern of cells in the pituitary gland serves as a further aid in the identification of the cells

Concerning the cellular site of STH, LTH and gonadotropic hormone production the present results are in agreement with most of the recent reports in the literature Of the two different groups of gonadotrophs the one (FSH cells) is mainly peripheral while the other (LH cells) is in the central part of the lobe, the latter differs somewhat from the thyrotropic cells in shape and distribution rendering differentiation possible Best differentiation between FSH and LH cells was obtained by the alcian blue technique at pH 0 2 In other staining methods these cells were stained about equally The affinity of the granules of these gonadotropic cells to basic dyes as well as to alcian blue at higher pH and basic fuchsin in Schiff staining was clearly weaker than that of the thyrotropic cells

Except on the basis of the differential staining characteristics the two acidophilic cell types the somatotropic and the LTH cells can also be identified due to their typical changes in various experimental conditions as described earlier

Estrogen treatment produces a great enlargement of the pituitary gland It has been found that the acidophilic cell group is activated by this treatment (Severinghaus 1939 Baker and Everett 1944, Meyer et al 1946) Purves and Griesbach (1952) identified the LTH cells as the acidophils which degranulate after castration and estrogen treatment Quantitative studies have shown a marked increase of the LTH content in the pituitary gland after estrogen treatment (Reece and Turner 1937 Meites and Turner 1948) and the same is found during lactation (Hurst and Turner 1942) LTH cells were few in normal male animals but were increased after castration a fact described also earlier (Hurst and Turner 1942, Lacour 1950 Dubois and Girod 1964) If a female mouse is castrated before sexual maturation the LTH cells fail to differentiate (Barnes 1962)

The site of corticotropic hormone production in the pituitary gland of

the rat is a very disputed subject and the demonstration of the possible cell type demands increased ACTH formation and secretion with the corresponding changes in cellular morphology The changes are not however always appropriate evidence for the origin of a certain hormone This is confirmed e g by the marked decrease of acidophilic cells after thyroidectomy or methylthiouracil feeding although it is well known that these cells do not take part in the production of TSH (Griesbach 1953) The cell type which in this study is suggested to be the ACTH cell greatly resembles that described by Herlant and his colleagues (Racadot and Herlant 1960 Pasteels and Herlant 1962 Herlant and Klatersky 1963 Quenum and Herlant 1964)

Quite specific staining techniques for the thyrotropic cells seem to be aldehyde fuchsin and aldehyde thionin methods It was found, however that the dye easily deteriorated during storage although it was stored at room temperature as recommended by Elftman (1959) Although the TSH cells were not stainable the aged dye solution was still capable of staining the neurosecretory material in the posterior lobe Identification of these cells was verified through the marked changes in the number of the cells found after the treatment with thyroxine or methylthiouracil as reported previously (Zeckwer et al 1935, Catchpole 1949, Purves and Griesbach 1951b Halmi 1952b Dhom and Tietze 1962 Dhom et al 1962)

HISTOCHEMISTRY OF CARBOXYLIC ESTERASES IN THE ADENOHYPOPHYSIS

LITERATURE REVIEW

Classification and characterization of carboxylic esterases

The *cholinester hydrolases* as an estera e group represent a highly inhomogeneous group of enzymes and many different variants have been reported (cf Svensmark 1965) Two types of *cholinesterases* (ChE) can be rather specifi ally separated acetyl cholinestera e or acetylcholine acetyl hydrolase (AChE) and non specific cholinesterase or acyl choline acyl hydrola e (ns ChE) Both types of these can be separated from non specific esterases (ns E) due to their su ceptibility to eserine at low concentration (10^{-6} M) AChE and ns ChE differ from each other in substrate specifity and i the inhibitory characteristics with respect to different organophosphate esters carbamate derivatives and quaternary ammonium salts (Aldridge 1963a Augustinsson 1960, Pearse 1961)

The distinction of *lipase* (glycerol ester hydrola e) from the other n Es is based on its ability to act upon esters of long chained fatty acids (C 8 and upwards) or on undi olved substrates and on the specific activation by bile acids Cholesterol esterase (Myers et al 1955) and a lipase in lung and adipo e tissue (Hulsmann et al 1964) have been differentiated from the pancreatic lipase

The *non specific esterases* (ns E) which hydrolyze short chained fatty acid esters were cla sified by Aldridge (1953b c) into organophosphorous resistent (A estera es, E r ns E) and sensitive (B esterases E s ns E) groups, the most used inhibitor for this distinction has been diethyl p nitrophenylphosphate (E600) A third type of enzyme called C e terase is differentiated from the E600 resistent group by its inability to hydrolyze organophosphorou compounds and by the activation of the hydrolysis with low concentrations of p chloromercuribenzoate (p CMB Bergmann et al 1957, Bergmann and Pinon 1958 1960) Gomori (1955) made further distinction in the E600 ensitive group on the ba is of the respon e to NaF and called the NaF resistent enzyme the pancreatic type and the NaF sensitive the hepatic type of esterase

A large number of *proteases and peptidases* have been described which are capable of hydrolyzing in addition to peptide bonds esters of substituted amino acids and peptides (Neurath and Schwert 1950 Wiggans et al. 1954 Johnston 1956, Myers et al 195 Martin and Axelrod 1957 Shippey and Binkley 1958, Lagunoff and Benditt 1964) It has been concluded that the esterases especially E600 resi tent forms as revealed by histochemical techniques may in fact include different intracellular protea es or

peptidases such as cathepsin C (Hess and Pearse 1958) It has become evident later however that the latter enzyme is distinct from any esterase activities hydrolyzing 5 bromoindoxyl acetate one of the most used substrates for E r ns Es (Vanha Perttula et al 1965) Some trypsin like (Glenner et al 1962 Hopsu and Glenner 1963a, b) and chymotrypsin like (Lagunoff and Benditt 1964, Riekkinen and Hopsu 1965) enzymes as well as acylases in different tissues (Hopsu and Glenner 1964 Hopsu et al. 1965a b) have been suggested to contribute to the hydrolysis of various naphthol esters

A variant of *carbonic anhydrase* has been described which is capable of hydrolyzing ester substrates, and a tentative name of D esterase has been suggested for it (Shaw et al 196 Tappan et al 1964) Electrophoretic separation of carbonic anhydrase isozymes from esterase activities has been however reported in erythrocyte hemolysates and rat kidney extracts (Hyyppa et al 1966)

Adenohypophyseal carboxylic esterases

Cholinesterases

In the human pituitary gland ns ChE was found localized mainly in fully granulated basophils but some activity was also present in chromophobes and in the colloid (Pearse 1952) The existence of AChE in the FSH cells of the pituitary gland of rabbit was shown by Dumont (1956a), no ns ChE activity could be demonstrated In the guinea pig pituitary gland, on the other hand ChE activity was mostly non specific (Dumont 1956b) This activity was also located in beta basophils (FSH cells) but butyrylthio choline was also hydrolyzed in extracellular colloid and capillary endothelial cells

Differences have been observed in the content of AChE and ns ChE in different parts of the pituitary gland of different animals (Koelle and Geesey 1961 Arvy 1961, 1962 1964, Arvy and du Mesnil du Buisson 1961 Kobayashi and Farner 1964) As a rule however both ChEs are located mainly in the paramedian zone of the anterior pituitary gland in mammals and in the cephalic part in the gland of the sparrow With biochemical methods only AChE was demonstrated in the anterior and posterior pituitary lobes of the beef (Pasetto 1958)

Non specific esterases

The first demonstration of carboxylic esterase activity in the anterior pituitary tissue of the rat was made by Leduc and Wislocki (1952) using α naphthyl acetate as substrate Using the tween technique of Gomori (1945) for the demonstration of esterase and lipase activity no hydrolysis was found in the rat pituitary gland by Verne and Hébert (1952) and Verne (1954) In human pituitary gland the same method gave a distribution which suggested that the lipase activity may be in close connection to the pituitary lipids and have some effect on the permeability of the cell membranes (Curri 1956) Rat adenohypophysis was classified as an organ of slight esterolytic activity toward 5 bromoindoxyl acetate by Barnett (1959)

Gomori (1955) found that anterior pituitary basophils contained an esterase activity which can be revealed using α naphthyl acetate α naphthyl propionate α naphthyl acrylate α naphthyl butyrate or naphthol AS acetate as substrates The activity is

totally inhibited by E600 (10^{-5} M), markedly inhibited by di isopropyl fluorophosphonate (DFP, 10^{-5} M), but not inhibited with NaF (0 5—2×10^{-3} M), it was slightly activated by lauryl sulfate

Esterase activity was found in human pituitary basophilic cells by Fand (1955) after paraffin embedding She however, could show that there is a group of basophils in the intermediate lobe and invading the posterior lobe which were devoid of α naphthyl acetate and naphthol AS acetate splitting activity Both cell types could be stained with PAS and aldehyde fuchsin Further studies on human material (Fand 1961) have demonstrated that the enzymatically active basophils of the anterior pituitary gland may be gonadotrophs and such cells are sometimes also invading the posterior lobe, especially in females

Further studies on human material showed the existence of two types of esterase which were resistent to eserine (Pearse 1956) One of them was located in the PAS positive "mucoid cells This activity was sensitive to E600 (10^{-5} M) and was thus classified as B esterase It was observed with acetate and butyrate esters of indoxyl as well as with α naphthyl acetate and naphthol AS acetate but not with indoxyl laurate These findings agree with those of Gomori (1955) in respect to the pancreatic type of hypophyseal esterase The possibility of the existence of two nearly identical enzyme activities in mucoid cells was suspected on the basis of different results obtained with fresh frozen sections and after dehydration and paraffin embedding The same group of basophils found by Fand (1955) to lack esterase activity was active in fresh frozen sections but could not be found after paraffin embedding It was suggested that the enzyme may occur in two different forms of which one was lost during the dehydration procedure

Ns E activity with different substrate and inhibitor characteristics was found in perisinusoidal cells (Pearse 1956) This enzyme activity utilizes only acetate esters of naphthol and indoxyl but not esters with longer chain length It increases during pregnancy and in other states with increased degranulation of basophilic mucoid' cells It was suggested that this esterolytic activity may be caused by an intracellular peptidase

In rat pituitary gland ns.E activity was histochemically demonstrated by Lojda (1960) and Lojda and Schreiber (1960 1964) With α naphthyl acetate and naphthol AS acetate as substrate maximum activity was observed in gonadotropic and thyrotropic cells Both E s ns E and E r ns E activities were present in these cells

Using 5 bromoindoxyl acetate as substrate Herlant and Grignon (1961) found esterase activity in the turtle pituitary gland in two different cell types somatotropic and lactotropic cells This activity was thought to be associated with the high phospholipid content of these two cell types An E r ns E has also been demonstrated in gonadotropic cells of various amphibian species (Wachtler and Pearse 1966)

Sobel (1962) observed that thyrotropic cells of the rat pituitary gland contained a diffuse cytoplasmic esterase as well as an E r ns E located identically with acid phosphatase probably in the lysosomal particles and in addition in the Golgi zone This activity was thought to be associated to the secretion of thyrotropic hormone

Studies on human embryos have shown that no esterase activity is present in Rathke s pouch either in 6 and 7 mm long embryos (McKay et al 1956) or 45 mm long embryos (Possi et al 1954 1957) Pearse (1956) suggested that both thyrotrophs and gonadotrophs are active in the fetal gland, the first appearance of the enzyme activity coincided with the beginning of the mucoprotein hormone secretion of these cells and

the enzyme activity was associated with the mucoprotein granules Jirá ek (1963) has also studied the estera e activity in the fetal pituitary gland of man and could dif ferentiate two different cell types which were active from the end of the third month of development In PAS positive peripheral and central cells an enzyme activity appears which is also found in paraffin embedded sections From the fourth month on another activity in chromophobe cells of the basophilic line became active but this activity was present only in fre h frozen sections These cells showed perinuclear activity and, in addition, there was a diffuse cytoplasmic reaction Cells covering the rest follicles in the intermediate lobe showed al o an esterase activity in the apical part of the cytoplasm

In addition to the basophils esterase activity has also been demonstrated in other hypophy eal cell types Thus Pear e (1956) demonstrates another estera e activity in perisinusoidal cells This enzyme activity corresponds to that found in pericytic cells and neurons of hypothalamic nuclei (Pepler and Pear e 1957 Pear e 1958) and in some cellular components of rat kidney (Hess and Pear e 1958) On the basis of activator and inhibitor tudies it was suggested that this may be an intracellular pep tida e probably cathep in C

The cellular di tribution of E s n E is mainly diffus or finely granular in basophilic and PAS positive cells in different animal species including man (Gomori 1955 Fand 1955 1961, Pearse 1956, Sobel 1962 Jirasek 1963 Lojda and Schreiber 1960, 1964) E r ns E has been found to reside near the Golgi zone (Pearse 1956 Sobel 1962 Jirásek 1963 Lojda and Schreiber 1960 1964) This activity is the only one observed after paraffin embedding (Pearse 1956) and it has been claimed that this activity is confined to small corpuscles which may be ly osomes (Pearse 1956 Pepler and Pearse 1957 Hess and Pearse 1958 Pear e 1961, Pearse and van Noorden 1963a b Sobel 1962 Lojda and Schreiber 1964 Wächtler and Pearse 1966)

Changes in experimental conditions

No reports have been published of variations in the ChE activity of the anterior pituitary cells in experimental conditions In human material the diffuse cytoplasmic ns E activity follows the excretory pattern' of the cells (Pearse 1956) During the increased activity of the mucoid cells the esterase activity is moved to the cellular periphery near the bordering peri inu oidal space Simultaneou ly increased activity is observed in pericytes and transfer of e terase to the e cells by phagocytosis was sug gested Studies of Jirásek (1963) do not confirm the increa ed esterase activity in human adenohypophysis in connection with the removal of the PAS positive granula from the cells but on the contrary increa ed esterase activity is connected with the ynthe is of PAS positive glyco and mucoprotein granules

In man increased granular perinuclear activity was found during pregnancy in acidophils, thyroidectomy cells had also the same type of activity (Pearse 1956) Thyroidectomy and feeding of methylthiouracil in rats increases the granular E r ns E activity (Pearse 1956 Sobel 1962 Lojda and Schreiber 1960 1964) Simultaneously a decrease in enzyme activity in other ' mucoid cells as well as a very marked increase in ' mucoid cells of the pars intermedia was reported (Pearse 1956)

Castration cells in rats have al o been found to contain increased amount of both

totally inhibited by E600 (10^{-4} M), markedly inhibited by di isopropyl fluorophosphonate (DFP, 10^{-4} M), but not inhibited with NaF (0 5—2×10^{-2} M), it was slightly activated by lauryl sulfate

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and they were all obtained from Sigma Chem Comp (Ohio USA) The final concentration of the substrate was ₄ mM Fast Garnet GBC (Edward Gurr Ltd London England) was used as coupling agent at a concentration of 5 mg/ml and no counter tain was used The optimal incubation times for the different substrates were as follows α NA, α NPr and N AS for 3—5 min α NB and α NV for 6—10 min at room temperature and α NC, N AS D and β NL for 30—60 min at 37 C When inhibitors were used 30 min preincubation at room temperature or at 37 C respectively preceded without substrate and coupling agent The inhibitor substances were al o present at the ame concentration in the final substrate solution

5 Bromoindoxyl acetate (5 BIA) was used as sub trate according to the method of Holt (1952, 1956 1958) and Holt and Withers (1958) The substrate solution was prepared according to Pearse (1961), but in addition the hydroly is was tested at a lower (0 5 mM) ferro and ferricyanide concentration (Shnitka and Seligman 1961) The incubation time was 30—60 min at room temperature The method of Wachstein et al (1961) was employed for the hydrolysis of thioacetic acid (TAA Fluka AG Buchs SG Switzerland) The incubation time was 60 min at 37 C

Modifiers for the classification of esterases

The inhibitor substances used in the present investigation are listed in Table III where also the routinely used concentration of the inhibitor for any sensitive enzyme is given Sodium taurocholate is also used to activate the hydrolysis of long chained fatty acid esters of α and β naphthol by a pancreatic type of lipase (Abe et al 1964) possibly present in pituitary tissue

Other modifier sub tances used in the studies were as follows Sodium p chloromercuribenzoate (p CMB Calbiochem Calif USA) at a concentration of 10 M was used with E600 (10^{-5} M) to study the presence of C type esterase activity (Bergmann et al 195, Bergmann and Rimon 1958 1960) The effects of Triton X 100 (Minor Lautenbourg France) on the histochemical reaction was tested at concentrations of 0 05 and 0 1 % (Allen et al 1965) Perazine (Peratsin®, Laake OY, Turku Finland) was used at 10 M concentration to inhibit phenothiazine sensitive estera es (Hulsmann et al 1964)

Results

Carboxylic esterases in normal rat pituitary sections

The intensities of the esterase activities in the different pituitary cell types are presented in Tables IV and V as obtained with different sub strate inhibitor combinations The intensities are expressed with signs ++++ +++ ++ +, and —, which mean the decreasing order of the enzymic reaction obtained Table IV summarizes the results for fresh sections and Table V for formalin fixed sections

Table III Inhibitors used in the differentiation and classification of esterases

Inhibitor	Abbre viation	Source	Concentra tion (M)	Sensitive enzyme	Reference
Eserine sulphate	Eserine	Nutr Biochem Corp Ohio USA	10^{-5}	ChE	Aldridge 1953a Gomori 1952
1 5 bis (4 trimethyl ammoniumphenyl) pentan 3 one diiodide	62 C 47	Wellcome Res Lab Beckenham, England	10^{-5}	AChE	Bayliss & Todrick 1956 Pepler & Pearse 1957
1 5 bis (4 allyl dimethyl ammoniumphenyl) pentan 3 one diiodide	284 C 51	Wellcome Res Lab Beckenham, England	10	AChE	Bayliss & Todrick 1956 Holmstedt 1957
Tetra isopropyl pyrophosphoramide	*iso* OMPA	Light & Co Ltd Colnbrook England	$10^{[illegible]}$	ns ChE	Aldridge 1953a Diegenbach 1965
Ethopropazine methosulphate	Lysivane	May & Baker Ltd Dagenham England	10^{-4}	ns ChE	Bayliss & Todrick 1956
Diethyl p nitrophenyl phosphate	E600	Mintacol® Bayer Leverkusen Germany	10^{-5}	E s ns E	Aldridge 1953a, b, c
Di isopropyl fluoro phosphonate	DFP	Light & Co Ltd Colnbrook, England	$10^{[illegible]}$	E s ns E	Aldridge 1953a b c
Sodium taurocholate	TC	British Drug Houses Poole, England	10^{-5}	ns E	Abe et al 1964
Sodium fluoride	NaF	E Merck AG Darmstadt, Germany	$10^{[illegible]}$	E s ns E hepatic type	Gomori 1955
Cupric chloride	Cu	E Merck AG Darmstadt Germany	10^{-4}	E r ns E A esterase	Pearse 1956 Bergmann et al 1957

Table IV Esterases in *fresh frozen sections* obtained with different substrate inhibitor combinations The inhibitor concentrations as given in Table III.

Substrate and inhibitor	Gonado troph	Thyro troph	Somato troph	Peri cytes	Capillary endo thelium	Cleft epi thelium
AcThC	—	—	—	—	+	+
iso OMPA	—	—	—	—	+	—
284 C 51	—	—	—	—	+	+
Eserine	—	—	—	—	+	—
E600	—	—	—	—	+	—
Lysivane	—	—	—	—	+	—
BuThC	—	—	—	—	+	+
iso OMPA	—	—	—	—	+	—
284 C 51	—	—	—	—	+	+
Eserine	—	—	—	—	+	—
E600	—	—	—	—	+	—
Lysivane	—	—	—	—	+	—
α NA	+++	+++	++	—	—	+
iso OMPA	+++	+++	++	—	—	+
284 C 51	+++	+++	++	—	—	+
Eserine	+++	+++	++	—	—	+
E600	—	—	—	—	—	—
DFP	—	—	—	—	—	—
NaF	—	—	—	—	—	—
Cu++	++	++	+	—	—	+
p CMB	+++	+++	++	—	—	+
TC	+	+	++	—	—	+
α NPr	+++	+++	+	—	—	++
iso OMPA	+++	+++	+	—	—	++
284 C 51	+++	+++	+	—	—	++
Eserine	+++	+++	+	—	—	++
E600	—	—	—	—	—	—
DFP	—	—	—	—	—	—
NaF	—	—	—	—	—	—
Cu++	++	++	+	—	—	+
p CMB	+++	+++	+	—	—	++
TC	+	+	+	—	—	+
α NB	+	+	±	—	—	+++
iso OMPA	+	+	±	—	—	+++
284 C 51	+	+	±	—	—	+++
Eserine	+	+	±	—	—	+++
E600	—	—	—	—	—	—
DFP	—	—	—	—	—	—
NaF	—	—	—	—	—	—
Cu++	+	+	—	—	—	++
p CMB	+	+	±	—	—	+++
TC	±	±	—	—	—	++

Table IV (continued)

Substrate and inhibitor	Gonado troph	Thyro troph	Somato troph	Peri cytes	Capillary endo thelium	Cleft epi thelium
α NA	—	—	—	—	—	++
α NC	—	—	—	—	—	—
N AS	—	—	—	—	—	—
N AS D	—	—	—	—	—	—
5 BIA	+	+	+	—	—	—
iso OMPA	+	+	+	—	—	—
284 C 51	+	+	+	—	—	—
Eserine	+	+	+	—	—	—
E600	—	—	—	—	—	—
DFP	—	—	—	—	—	—
NaF	—	—	—	—	—	—
Cu ++	—	—	—	—	—	—
p CMB	+	+	+	—	—	—
TC	—	—	—	—	—	—
TAA	—	—	—	—	—	—

Hydrolysis of thiocholine esters

In *unfixed sections* the hydrolysis of both AcThC and BuThC was nearly absent except for some weak activity in capillary endothelial cells and in cells lining the pituitary cleft after 24 hours of incubation

In *fixed sections* both these activities were also present but additionally some activity was obtained in some large cells in the central part of the gland These cells were polygonal (Figs 7 and 10) and clearly reminiscent of the TSH cells in histological studies With both substrates the reaction product was mainly diffuse but small granules were also found The reaction in the cleft epithelium was diffuse, but the weak activity in capillary endothelium seemed to be mainly granular (Figs 8 and 11) The intensity of these reactions was quite weak in comparison to the activities found in the posterior lobe long incubation times were necessary

The hydrolysis of both substrates was totally inhibited in the polygonal cells by eserine (Fig 8) iso OMPA (Fig 11) and E600 (Fig 12), but not by 284 C 51 (Fig 9) The diffuse activity in the cleft epithelium had identical characteristics which are typical for the ns ChE

The weak granular activity following the sinusoids was resistent to eserine (Fig 8) iso OMPA (Fig 11) as well as to E600 (Fig 12) Thus this reaction cannot be ascribed to either AChE or ns ChE

Table V Esterases in *formalin fixed sections* obtained with different substrate-inhibitor combinations. The inhibitor concentrations as given in Table III

Substrate and inhibitor	Gonadotroph	Thyrotroph	Somatotroph	Pericytes	Capillary endothelium	Cleft epithelium
AcThC	—	+	—	—	+	++
iso-OMPA	—	—	—	—	+	—
284 C 51	—	+	—	—	+	++
Eserine	—	—	—	—	+	—
E600	—	—	—	—	+	—
Lysivane	—	—	—	—	+	—
BuThC	—	+	—	—	+	++
iso-OMPA	—	—	—	—	+	—
284 C 51	—	+	—	—	+	++
Eserine	—	—	—	—	+	—
E600	—	—	—	—	+	—
Lysivane	—	—	—	—	+	—
α NA	++++	+++	++	+++	++	++
iso-OMPA	++++	+++	++	+++	++	++
284 C 51	++++	+++	++	+++	++	++
Eserine	++++	+++	++	+++	++	++
E600	++	++	+	—	++	—
DFP	++	++	+	—	—	—
NaF	++	++	+	—	++	—
Cu	++	++	+	++	—	++
p-CMB	+++++	+++	++	++	++	++
TC	++++	+++	++	++	++	++
α NPr	++	++	+	+++	—	+++
iso-OMPA	++	++	+	+++	—	+++
284 C 51	++	++	+	+++	—	+++
Eserine	++	++	+	+++	—	+++
E600	+	+	—	—	—	—
DFP	+	+	—	—	—	—
NaF	+	+	—	—	—	—
Cu^{++}	+	+	+	++	—	+++
p-CMB	++	++	+	+++	—	+++
TC	++	++	+	+++	—	+++
α NB	+	+	—	++++	—	++++
iso-OMPA	+	+	—	++++	—	++++
284 C 51	+	+	—	++++	—	++++
Eserine	+	+	—	++++	—	++++
E600	—	—	—	—	—	—
DFP	—	—	—	—	—	—
NaF	—	—	—	—	—	+
Cu^{++}	+	+	—	++++	—	++++
p-CMB	+	+	—	++++	—	++++
TC	+	+	—	++++	—	++++

Table IV (continued)

Substrate and inhibitor	Gonadotroph	Thyrotroph	Somatotroph	Pericytes	Capillary endothelium	Cleft epithelium
α NA	—	—	—	—	—	++
α NC	—	—	—	—	—	—
N AS	—	—	—	—	—	—
N AS D	—	—	—	—	—	—
5 BIA	+	+	+	—	—	—
iso OMPA	+	+	+	—	—	—
284 C 51	+	+	+	—	—	—
Eserine	+	+	+	—	—	—
E600	—	—	—	—	—	—
DFP	—	—	—	—	—	—
NaF	—	—	—	—	—	—
Cu^{++}	—	—	—	—	—	—
p CMB	+	+	+	—	—	—
TC	—	—	—	—	—	—
TAA	—	—	—	—	—	—

Hydrolysis of thiocholine esters

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The hydrolysis of both substrates was totally inhibited in the polygonal cells by eserine (Fig 8) *iso* OMPA (Fig 11) and E600 (Fig 12) but not by 284 C 51 (Fig 9) The diffuse activity in the cleft epithelium had identical characteristics which are typical for the ns ChE

The weak granular activity following the sinusoids was resistent to eserine (Fig 8) *iso* OMPA (Fig 11) as well as to E600 (Fig 12) Thus this reaction cannot be ascribed to either AChE or ns ChE



Fig ~ AcThC Γixed section ×240
Fig 8 AcThC Fixed section Eserine 10   M ×240
Fig 9 AcThC Fixed s ction 284 C 51 10   M ×240
Fig 10 BuThC Fixed section ×240
Fig 11 BuThC Fixed section *Iso* OMPA 10   M ×240
Fig 12 BuThC Fixed section. E600 10   M ×240
Fig 13 α NA. Unfixed section ×150
Fig 14 α NA. Unfixed section Triton X 100

clearly diminished activity was present in these cells after sodium taurocholate treatment (Fig 15)

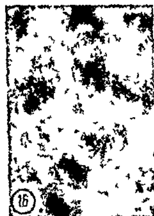
In addition to these cells a weaker activity was found in almost all other cells. Finely granular deposits of the reaction product was in the cytoplasm increasing towards the cell periphery. The over all result in the histological section was a net or honeycomb like picture (Figs 13 and 15). This type of activity is most clearly present in somatotropic cells and it has the same affector characteristics as those previously reported for esterase activity in gonadotropic and thyrotropic cells. When Triton X 100 was included in the incubation medium at 0.1 % concentration no enzymic activity was found in the anterior pituitary cells. At 0.05 % concentration the gonadotropic and thyrotropic cells were still devoid of activity, but an intense net like activity was shown corresponding mainly with the cellular periphery of the somatotropic cells (Fig 14), under which it is also found in Table IV and V. The same activity was also quite well preserved in the presence of taurocholate (Fig 15)

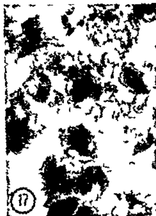
The epithelium covering the residual cleft between anterior and intermediate lobes showed some activity with the same affector characteristics as the former cell types (Fig 49 black arrow). Triton X 100 (0.05 %) and sodium taurocholate were only slightly inhibitory. The activity with α-NA as substrate was however rather weak in comparison to some other substrates reported later

In *formalin fixed sections* a very intense esterase activity was obtained after a much shorter incubation time than that used in fresh frozen sections. This indicated that at least some of the esterase activities were soluble and could not be revealed in unfixed sections. This was further confirmed by the inhibitor studies

The most active cells turned out to be again the gonadotropic FSH and LH cells but the polygonal thyrotropic cells showed also an intense activity. The activity was located in these cells near the nucleus and the reaction appeared first as course granules. After a longer incubation time the reaction products tended to cover also the nuclear area (Fig 16). This activity was clearly augmented by p CMB (Fig 17). F600 and DFP were without effect on this coarse granular activity

An L r ns F activity was also to a smaller degree observed in numerous other cells types. The most active cells were somatotropic cells but ACTH cells also showed this type of esterase. The reaction was granular and most active perinuclearly. No differences were observed in the inhibitor studies in respect to the coarse granular activity in gonadotropic and thyrotropic cells









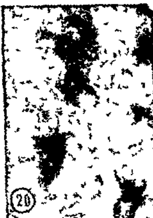










Fig. 16. α NA. Fixed section. ×240

Fig. 17. α NA. Fixed section. pCMB 10^{-4} M. ×240

Fig. 18. α NA. Fixed section. CuCl 10^{-4} M. ×240

Fig. 19. α NIr. Unfixed section. ×120

Fig. 20. α NIr. Fixed section. ×240

Fig. 21. α NIr. Fixed section. F600 10^{-4} M. ×240

Fig. 22. α NIr. Fixed section. CuCl 10 M. ×240

Fig. 23. α NIr. Fixed section. Triton X 100 0.1%. ×240

Fig. 24. α NIr. Fixed section. F600 10^{-4} M and pCMB 10^{-4} M. ×240

The sinusoidal epithelial cells also consistently showed a fine granular E ins E activity. This differed from the other E ins E's due to its sensitivity to DFP and Cu^{+} (Fig 18). The epithelial cells of the residual cleft showed more intense activity in fixed sections than in fresh frozen sections. This activity had the same inhibitor characteristics as described and thus seems to represent an E s ns E activity. The other E s ns E activities found in fresh frozen sections cannot be observed in fixed sections due mainly to their inactivation in the fixative.

Another esterolytic activity was revealed which was not evident in unfixed sections. This activity resides in fusiform cells around the sinusoidal capillaries. This cell corresponds in its appearance and location to the perisinusoidal cell. The enzymically active cells in the normal rat were preferentially located in areas with numerous active FSH cells, i.e. in the sex zone. This activity was sensitive to E600, DFP and NaF, it thus exhibited the same characteristics as the esterase in fresh sections. Triton X 100 at 0.05 and 0.1 % concentration had some activating effect on this esterase. Cu does not inhibit this enzyme and the active pericytic cells are thus differentiated from capillary endothelial cells which have Cu^{+} sensitive activity (Fig 18).

α Naphthyl propionate. — α NPr revealed nearly identical distribution and inhibitor characteristics of hydrolytic activity in *fresh frozen sections* as α NA. The results obtained with Triton X 100 are also in agreement with the findings obtained with α NA. All of these characteristics suggest that α NA and α NPr are hydrolyzed by the same enzymes. Some relative intensity differences between the different sites of activity were however observed. Thus the honeycomb-like distribution of hydrolytic activity turned out to be weaker in comparison to the esterase activity in gonadotropic and thyrotropic cells (Fig 19), while, on the contrary, a much more appreciable activity was present in the cleft epithelium.

In *formalin fixed sections* significant differences could be found with α NPr in comparison to α NA. The most active hydrolysis of α NPr occurred in the pericytic cells (Fig 20). When this activity was inhibited by E600 (Fig 21) it became evident that this substrate was also split by a parenchymal E r ns E. This reaction which was much weaker than with α NA was present in gonadotropic and thyrotropic cells. This activity was also resistent to NaF and only partially inhibited by DFP. Cu^{++} at higher concentration (10^{-3} M, Fig 22) was able to extinguish all of this coarse granular activity, but the E600 sensitive pericytic activity was still noticed although markedly decreased. In the presence of E600 and p-CMB simultaneously the pericytic activity was totally inhibited while a clear

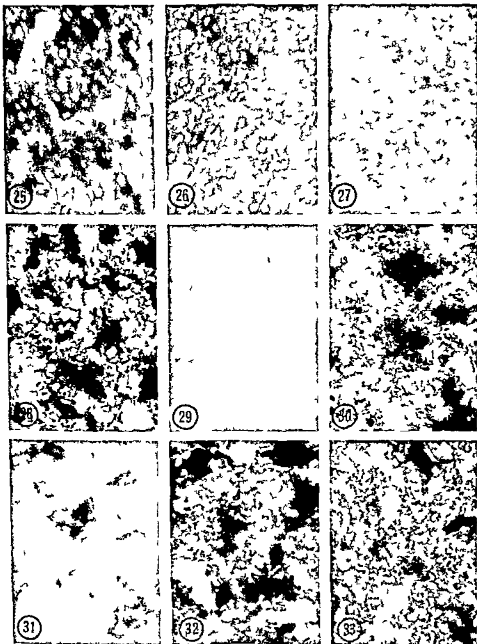


Fig 25 α NB Unfixed section. ×150
Fig 26 α NB Unfixed section. Eserine 10 M ×150
Fig 27 α NB Unfixed section Sodium taurocholate 10 M ×150
Fig 28 α NB Fixed section ×240
Fig 29 α NB Fixed section E600 10^{-5} M ×240
Fig 30 α NB Fixed section Sodium taurocholate 10 M ×240
Fig 31 α NV Fixed section ×240
Fig 32 α NV Fixed section Triton X 100 0 1 % ×240
Fig 33 α NC Fixed section Triton X 100 0 1 % ×240

activation of the granular perinuclear activity in gonadotropic and thyrotropic cells was observed. The same kind of activity in somatotropic and ACTH cells was also visualized by this method (Fig 24).

The pericytic activity was clearly activated by the presence of Triton X 100 (0.1 %), but no apparent effect on the E600 resistent parenchymal cell activity was revealed (Fig 23).

A very intense esterase activity was shown in the epithelial cells on both sides of the pituitary cleft with α NPr. This activity was totally inhibited by E600, NaF as well as by DFP but remained unaffected by eserine. These findings are consistent with those obtained with α NA as substrate, but this enzyme clearly preferred α NPr.

α Naphthyl butyrate. — In *fresh frozen sections* the hydrolysis of α NB by the gonadotropic and thyrotropic cells as well as the honeycomb-like activity was slight in fresh frozen sections, but using longer incubation times, a similar reaction was obtained as that with the previous substrates (Fig 25). Very intense activity was always obtained in the pituitary cleft epithelium also in fresh frozen sections. All these activities were sensitive to E600, DFP and NaF but resistent to eserine (Fig 26); the activity in gonadotropic and thyrotropic cells was almost totally inhibited by sodium taurocholate (Fig 27).

In *formalin fixed sections* the most prominent activity shown with α NB was located in the pericytic cells (Fig 28). This intense hydrolytic activity almost overshadowed the slight activity in gonadotropic and thyrotropic cells. α NB was found to be the preferred substrate for this pericytic activity. This substrate gave also a marked hydrolytic activity in the cleft epithelial cells (Fig 50). All these activities were sensitive to E600 (Fig 29), DFP and NaF. Sodium taurocholate had no apparent effect on the pericytic activity (Fig 30), while Triton X 100 (0.1 %) clearly enhanced this activity.

α Naphthyl valerate and α Naphthyl caprylate. — In *fresh frozen sections* α NV showed still weaker activity than α NB and with α NC no activity was present at all.

In *formalin fixed sections* α NV (Fig 31) was hydrolyzed by an activity in the pericytic cells with equal inhibitor characteristics as shown using α NB as substrate. The cleft epithelium also had a strong activity with α NV but α NC gave a negative result. When α NV (Fig 32) and α NC (Fig 33) were used with Triton X 100 (0.1 %) the activity was much increased in both the pericytic and the cleft epithelial cells. This effect seems to be due, at least partly, to a better solubilization of the substrates.

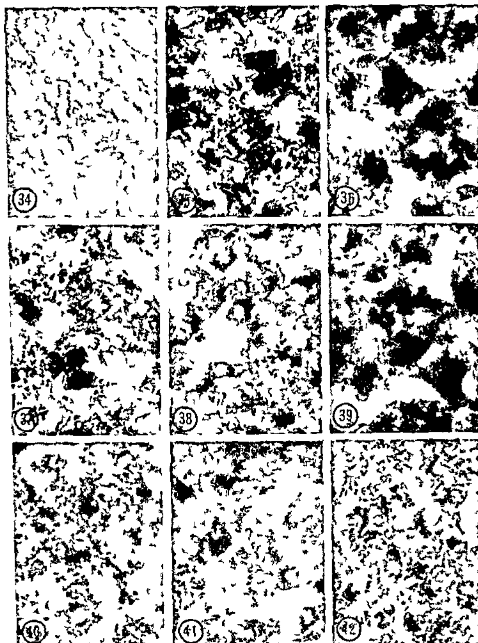


Fig 34 N AS Unfixed section ×150
Fig 35 N AS Fixed section ×240
Fig 36 N AS Fixed section *p CMB 10⁻⁴ M* ×240
Fig 37 N AS Fixed section E600 10⁻⁵ M ×240
Fig 38 N AS Fixed section. DFP 10 M ×240
Fig 39 N AS Fixed section Triton X 100 0 05 % ×240
Fig 40 N AS D Fixed section ×240
Fig 41 T A A Fixed section ×240
Fig 42 T A A Fixed section F600 10⁻⁴ M ×240

Naphthol AS acetate — In *fresh frozen sections* no or a very slight activity was observed after a long incubation time (Fig 34) On the contrary in *formalin fixed sections* a very intense activity was shown in different cell types (Fig 35) A strong coarse granular activity was found in gonadotropic and thyrotropic cells and a weaker one in somatotropic and most likely in corticotropic cells The esterase activity in all these cells was perinuclearly located Capillary endothelial cells showed also a weak granular activity No hydrolysis was present in the pericytes or in the cleft epithelial cells in the pituitary gland of the control rat

Some activation of the granular reaction was observed in the presence of p CMB (Fig 36) All the activities obtained were resistent to E600 (Fig 37) NaF and eserine DFP (Fig 38) had some inhibitory effect on the granular parenchymal cell activity and almost totally inhibited the hydrolytic reaction in the capillary endothelial cells Cu^{++} also decreased this latter activity Triton X 100 at 0 05 % concentration had no apparent effect (Fig 39), but 0 1 % concentration caused some inhibition

Naphthol AS D chloroacetate — In *fresh frozen sections* no activity was obtained with N AS D in any cells of the anterior pituitary gland

In *formalin fixed sections* both gonadotropic and thyrotropic cells showed an equal and granular, but weak activity, in their cytoplasm when N AS D was used as substrate (Fig 40) Weak activity was also present in capillary endothelial cells These activities were resistent to E600 and NaF

β Naphthyl laurate — No activity was ever obtained with β NL either in the presence or absence of any affector including sodium taurocholate The result was also negative in the presence of Triton X 100 in all concentrations tested

Thioacetic acid — TAA as substrate revealed no activity in *fresh frozen sections* In *formalin fixed sections* a large number of granular dye deposits were present in different cell types (Fig 41) The most active cells were again both types of gonadotropic cells as well as the thyrotropic cells but additional activity was present in almost every other parenchymal cell except the chromophobic ones Thioacetic acid esterase activity was also shown in the epithelial cells of the pituitary cleft (Fig 51) In these cells the activity was mainly diffuse or finely granular The coarse granular activity in different parenchymal cells as well as in the capillary endothelial cells was resistent to E600 (Fig 42) and NaF as well as to eserine thus the same characteristics were obtained as with N AS as substrate The esterase activity in the cleft epithelial cells was sensitive to E 600 and NaF but resistent to eserine, it may be identical with the enzyme preferring α NB as substrate

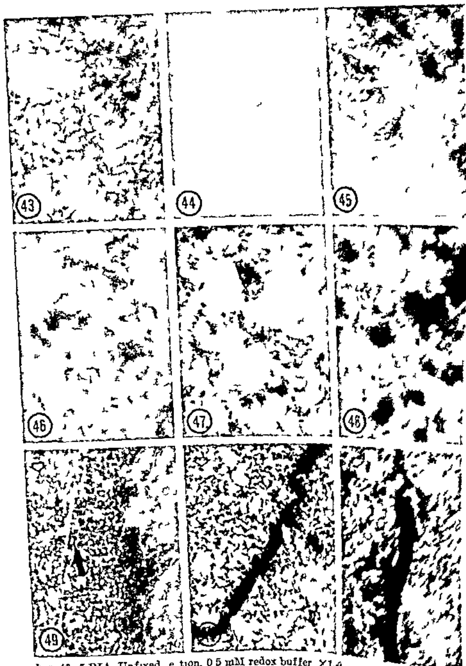


Fig 43 5 BIA Unfixed section, 0 5 mM redox buffer ×1..

Fig 44 5 BIA Unfixed section 5 mM redox buffer ×1..

Fig 45 5 BIA Unfixed section 0 5 mM redox buffer E... ×...

Fig 46 5 BIA Fixed section, 0 5 mM redox buffer ×240

Fig 4 5 BIA, Fixed section 5 mM redox buffer ×240

Fig 48 5 BIA Fixed section 0 5 mM redox buffer p CMB ... ×...

Fig 49 α NA Unfixed section Black arrow shows the ... adenohypophysis ×60

Fig 50 α NB Fixed section Open arrow shows the ad... ×...

Fig 51 T... Fixed section Open arrow shows the a... ×...

5 Bromoindoxyl acetate — Using 5 mM concentration of ferro and ferricyanides no activity could be obtained in *fresh frozen sections* (Fig 44) When the ferro ferricyanide redox buffer concentration was reduced to 0 5 mM, esterase activity was present in almost every cell, but most active were, however the gonadotropic and thyrotropic cells In Fig 43 the strongest activity is shown in the TSH cells around the sinusoids This activity was finely granular and distributed all over the cytoplasm but left the nuclei free It was inhibited by E600 and NaF, but was unaffected by eserine (Fig 45) It seems likely that these activities correspond to those obtained in fresh frozen sections with α NA and α NPr as substrate

In *formalin fixed sections* very intense coarse granular, perinuclear activity was present both in gonadotropic and thyrotropic cells when the original 5 mM concentration of ferro ferricyanide redox buffer was used in the incubation medium (Fig 47) The histochemical reaction totally resembled that obtained with N AS as substrate When the redox buffer concentration was lowered to 0 5 mM an additional fine granular activity was present in these cells as well as in a number of other cells Under these conditions the capillary endothelial cells showed also the same kind of fine granular activity (Fig 46) All these hydrolytic activities were resistent to E600 but the capillary endothelial activity was markedly inhibited by Cu^{++} and DFP No activity was present in the pericytes or in the cleft epithelium The effect of p CMB was clearly activating on the coarse granular, perinuclear activity in different cell types (Fig 48)

Changes in experimental conditions

Studies on the esterases in normal rat pituitary gland have shown that the ns Es can be grouped into E s ns E and E i ns E activities The former were present both in fresh frozen and in formalin fixed sections, while the latter were revealed only after fixation N AS was the preferred substrate for the E600 resistent activities but α NA α NPr, and α NB must be used for an appropriate demonstration of the different E s ns Es These findings are thus applied in the studies concerning the changes in the different esterases due to various experimental states

Cholinesterases

The hydrolysis of AcThC and BuThC was so weak that no precise estimations of an increase or decrease in various experimental conditions could be made This is true in both the activity in polygonal TSH cells as

well as the fine granular activity in capillary endothelial cells which was resistent to eserine The ns ChE in thyrotropic cells was also present in young rats

E 600 sensitive non specific esterases

The hydrolysis of α NA as well as α NPr was greatly enhanced in both types of gonadotropic cells after castration when the activity was followed in fresh frozen sections (compare Fig 52 to Fig 53) As a rule this activity is evenly distributed all over the cytoplasm but a longer time after the castration when signet cells appeared it was evident that the hydrolytic activity was absent in the area of the vacuole In such cases the enzymic reaction was more coarse granulated and perinuclearly located The same enzymic activity was also increased during pregnancy, but in this case the most active cells were the centrally located LH cells

The esterase activity in gonadotropic cells as visualized with α NA and α NPr was greatly decreased after the treatment with estrogen The enzymic activity was nearly homogeneous in intensity and very weak in the greatest part of the pituitary gland The only exception was the TSH cells which after this treatment were clearly found as a separate group of cells in the central part of the pituitary gland (Fig 54) In conditions when a large number of gonadotropic cells with a very intense esterase activity was present it was sometimes difficult to distinguish TSH cells from these

After progesterone treatment the esterase activity in the central gonadotropic or LH cells was decreased only to a small degree and remained unaltered in all other cell types Testosterone treatment on the contrary, caused a marked increase of esterase activity in FSH cells, but simultaneously the LH cells seemed to be almost devoid of this type of hydrolytic activity

The prominent FSH cells which were located in the periphery of the gland in young rats of about one month of age showed an intense activity with α NA (Figs 55 and 56) and α NPr, however, the reaction was nearly absent with α NB

Changes in the honeycomb like activity in different cell types was also variable in different conditions During increased gonadotropic secretion such as after castration this background activity seemed to be weaker This activity was very intense in the young rat pituitary (Fig 55) where it is mainly found in somatotropic cells The activity was always near to the cell membrane and the central perinuclear area was weaker in activity (Fig 56)

Thyroxine treatment decreased the esterase activity in TSH cells while the contrary was true after methylthiouracil treatment (Fig 57) The background activity representing mainly the somatotropic cells was however, much weaker in the latter case The thyrotropic cells were also easily separable from the gonadotropic cells due to the more intense activity in the polygonal cells No change from the control state however, occurred in the esterase content of the gonadotropic cells

During lactation the E s ns E in both gonadotropic cells was minimal and only some weak activity was present in somatotropic cells The lactotropic cells showed very little or none of this type of activity The same finding was valid also in other states of increased lactotropic hormone synthesis or secretion such as after estrogen or reserpine treatment

Correlation of these E s ns E activities in formalin fixed sections appeared to be impossible for a variety of reasons The most difficult task was to differentiate them from the E i ns E present in the same cells, but the E s ns E activities in the parenchymal cells were also markedly inactivated during the formalin fixation

After castration in both sexes the pericytic activity in the area of the TSH cells was markedly increased and extended to the central area of the pituitary gland (compare Fig 58 to 59) These cells were surrounding the enlarged gonadotropic cells but were not continuous like the sinusoidal capillary walls When castration cells appeared, the cytoplasmic activity near the cellular membrane was totally fused with the much greater pericytic activity present when α NA or α NPr were used as substrates These two activities are not, however, identical, since they differ from each other in substrate specificity The E s ns E in parenchymal cells is probably identical with that found within the same cells in unfixed section This activity is also markedly inactivated by formalin and becomes covered by the E i ns E The pericytic activity was resistent to formalin fixation and was best shown by α NB When the pituitary glands of young rats were fixed and the distribution of the hydrolytic activity against α NB was tested it was shown that only some isolated pericytic activities were found they were not situated near the TSH cells which presumably are actively secreting

No consistent change in the E s ns E activity in any parenchymal cell type was observed either during cortisone treatment or after adrenalectomy

The strong E s ns E activity towards α NPr and α NB and to a lesser degree to α NA and TAA in the cleft epithelium was best followed in formalin fixed sections It was found also in young rats but the activity was quite weak This activity was markedly increased after castration

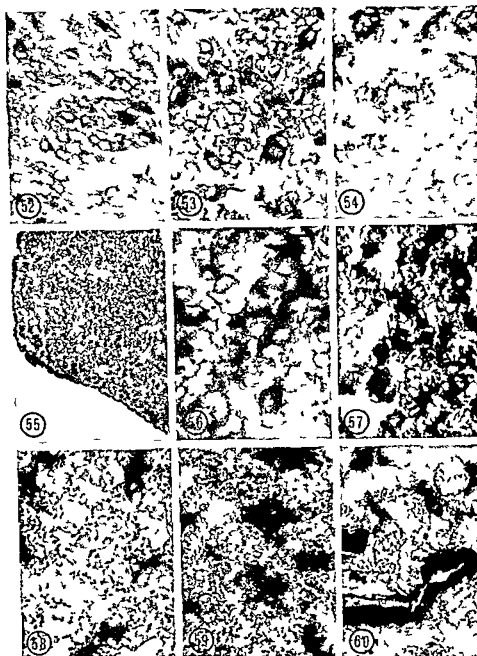


Fig 52 α NA Control female rat Unfixed section ×150
Fig 53 α NA Female rat castrated 30 days earlier Unfixed section ×150
Fig 54 α NA Female rat after 30 days treatment with estrogen. Unfixed section. ×150
Fig 55 α NA 30 days old male rat Sagittal unfixed section ×60
Fig 56 α NA Edge area of the Fig 55 ×240
Fig 57 α NA Male rat after 30 days treatment with methylthiouracil Unfixed section ×150
Fig 58 α NB Control male rat Fixed section ×240
Fig 59 α NB Male rat castrated 30 days earlier Fixed section ×240
Fig 60 α NB [illegible]

(Fig 60) some increase was also found after adrenalectomy Estrogen and cortisone treatment decreased this activity but progesterone and testosterone were without effect No apparent change was either shown after thyroxine or methylthiouracil treatment

E600 resistent non specific esterases

The strong E r ns E activity obtained with α NA (Fig 61) or N AS (Fig 64) was much increased in the cytoplasm of both LH and FSH cells after castration (Figs 62 and 65) This augmentation of the activity was apparent 15 days after gonadectomy Estrogen treatment markedly decreased the granular E r ns E in both gonadotropic cell types the most active cells were then thyrotropic and somatotropic cells (Figs 63 and 66) Testosterone treatment caused no apparent change in the hydrolytic activity of either gonadotropic cell type but the activity in somatotropic cells was somewhat augmented

In young female rats very intense activity was confined to the centrally situated angular cells these are presumably actively secreting TSH cells but the peripheral cells having very much E s ns E in fresh frozen sections were lacking this E r ns E almost totally Between the active TSH cells other smaller cells with very intense activity were observed These were mainly growth hormone secreting cells (Fig 67) The degranulated somatotropic cells of the methylthiouracil fed rats showed a moderate coarse granular activity The thyrotropic cells (Fig 68) showed an increased activity but the E r ns E in gonadotropic cells was much decreased during methylthiouracil feeding

The E r ns E was also increased during pregnancy in the central gonadotropic cells and this activity soon decreased after delivery During the first days after delivery the E r ns E in gonadotropic cells decreased rapidly but a similar activity was found in pericytic cells (Fig 69) These cells lost this activity about ten days after lactation This suggests that the enzymic protein is expelled from the gonadotropic cells and is picked up by the pericytic cells which have phagocytic properties The lactating rat pituitary has also very much diminished E r ns E in the gonadotropic cells and quite weak activity seems to be connected to the LTH cells

Adrenalectomy had only a slight effect on the E r ns E but a moderate increase in the hydrolytic reaction in ACTH cells could be constantly obtained After cortisone treatment the histochemical esterase reaction was seemingly augmented This effect was not caused by an increase in esterase activity in any special cell type but was the result of the tighter packing of the active gonadotropic cells in the shrunken pituitary gland

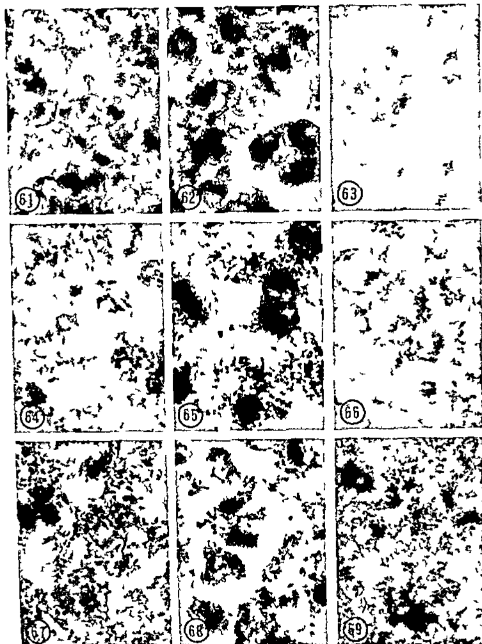


Fig 61 α NA Control female rat Fixed section ×240
Fig 62 α NA Female rat castrated 60 days earlier Fixed section ×240
Fig 63 α NA Female rat after 60 days treatment with estrogen Fixed section ×240
Fig 64 N AS Control female rat Fixed section ×240
Fig 65 N AS Female rat castrated 60 days earlier Fixed section ×240
Fig 66 N AS Female rat after 60 days treatment with estrogen. Fixed section ×240
Fig 67 N AS 30 days old female rat Fixed section ×240
Fig 68 N AS Male rat after 60 days treatment with methylthiouracil. Fixed section ×240
Fig 69 N AS Female rat 5 days after delivery Fixed section ×240

Discussion

Cholinesterases

The esterase activity in the thyrotropic cells had all the characteristics of ns ChE because it was inhibited by eserine, iso OMPA and ethopropazine at 10^{-5} M concentration Since this enzymic activity was found only after formalin fixation, it is probably solubilized during the long incubation time used The intracellular ChE activity is mainly bound to the ergastoplasmic reticulum as revealed by electron microscopic (Torack and Barrnett 1962 Mori et al 1964) and subcellular tissue fractionation studies (Underhay et al 1956, Aldridge and Johnson 1959 Hanzon and Toschi 1959 Toschi 1959)

Ns ChE as well as AChE are known to hydrolyze also naphthol and indoxyl esters (Pearse 1961) The contribution of the ns ChE activity to the high hydrolysis rate of these substrates in the TSH cells in the present investigation seems to be meager, because the effect of eserine was minimal

The other enzymic activity, which hydrolyzes thiocholine esters is situated perisinusoidally and is not inhibited by eserine or E600 it is neither AChE nor ns ChE Such an enzymic activity has been previously demonstrated in the endothelial lining of the hypothalamic blood vessels of rat but not in hedgehog ferret, or monkey this enzyme does not hydrolyse 5 BIA (Holmes 1961) In the present study this activity could not be clearly correlated with the enzymic activity obtained with other substrates but α NA and 5 BIA were also hydrolysed by an E r ns E activity in the capillary endothelial cells The actual site of the enzymic activity of this type may also be in the sympathetic ground plexus or secretory motor end plexus (Metuzals 1958) although available studies do not prove the existence of such an innervation in the pars distalis (Green 1951 Harris 1955)

E600 sensitive non specific esterases

The present histochemical study shows that all E s ns Es in the parenchymal pituitary cells are optimally demonstrable in fresh frozen sections This indicates that they are bound to some cellular particles In studies concerning the distribution of the esterase activity in the subcellular fractions of rat and mouse liver (Novikoff et al 1953 Underhay et al 1956 Carruthers and Baumler 1961 Carruthers et al 1965) and rat brain (Aldridge and Johnson 1959) E s ns E activity was mostly present in the

microsomal fraction Studies of Chaveau et al (1962) showed that the site of the esterases was in the membranes present in this fraction

Nonionic detergents, e g Triton X 100 are frequently used for the liberation of the lysosomal enzymes (Wattiaux and de Duve 1956) The esterases of the microsomal particles have also been solubilized by anionic detergents (sodium deoxycholate sodium lauryl sulfate sodium cetyl sulfate, sodium dodecyl benzene sulfonate) or by the nonionic detergents (Lubrol W and Tween 80 Carruthers and Baumler 1962) The effect of Triton X 100 on the histochemical esterase reaction in the fresh frozen pituitary section varied somewhat depending on the concentration of the detergent At 0 1 % concentration with 30 min preincubation all hydrolytic activity was lost Using lower concentrations some activity was still present in the cell periphery This difference suggests the presence of two different types of enzymes equal in affector characteristics but differing somewhat in intracellular location and substrate specificity

The marked increase found in the E s ns E activity in gonadotropic cells after castration and in thyrotropic cells after methylthiouracil treatment shows that this enzyme activity is connected to the increased production and/or release of the respective hormones Ribosomes aggregated in the endoplasmic reticulum have been found electron microscopically, indeed, in increasing amounts in gonadotropic cells after castration (Girod et al 1964 Yoshimura and Harumiya 1965) as well as in thyrotropic cells after thyroidectomy (Farquhar and Rinehart 1954 Cardell 1964)

The effect of estrogen and testosterone on the esterolytic activity in the gonadotropic cells is markedly different Estrogen treatment causes almost total depletion of the esterase activity in both LH and FSH cells but testosterone decreases only that in the LH cells and at the same time significantly increases the FSH cell activity These findings are in agreement with the studies concerning the effect of estrogen and testosterone on the synthesis of LH and FSH by the rat pituitary Estrogen has been shown to decrease both the FSH (Paesi et al 1955 van Rees 1961) and LH production (Greep and Chester Jones 1950) while testosterone increases the production of FSH (Hoogstra and Paesi 1957, Paesi et al 1959) and decreases that of LH (Paesi et al 1958)

The site of the other E s ns E was mainly in the peripheral part of the somatotropic cells although numerous other cell types showed this type of activity Triton X 100 was able to solubilize this activity too but at higher concentrations This may mean that the site of binding of this enzyme in the molecular cellular particles may be different In increased somatotropic production as could be anticipated in the young rats this

activity was found to be strong, and an apparent contribution of this enzymic activity to the hormone synthesis is thus likely.

The contribution of esterase activity to the release mechanism of the hormone granules must also be taken into account since the intracellular enzymic activity may dissolve the limiting membrane of the hormone granules by their transfer through the cell membrane (Yoshimura and Harumiya 1965). Methylthiouracil treatment discharges the granules of the somatotropic cells but it simultaneously depresses the hormone production. In this state the esterase activity was also found to be decreased. This suggests that also in this cell type the esterase activity is probably connected to the production of the hormone and not to its release. Since the weak E s ns E in other parenchymal cells showed no consistent changes during experimental conditions their role in the hormone production remains unclear.

The pericytic E s ns E found only in formalin fixed sections cannot be taken as an enzymic activity derived from the parenchymal cells by excretion because it had clearly differing substrate specificity. The pericytic cells are known to possess phagocytic properties (Cappell 1929) and it is thus to be expected that different hydrolytic enzymes are present. Rinehart and Farquhar (1955) and Farquhar (1961) suggested that these cells probably play an active role in the mechanism of hormonal transfer from the parenchymal cells into the sinusoids. These histiocytic cells may also be active in the phagocytic, digestive, and assimilative function toward the cytoplasmic debris from the parenchymal cells. To this function may belong also the capture of the remnants of the hormone granules and carrier proteins as well as the preparation of some nutritive material for the hormone producing cells. The enzymic activity found in these cells may be active in these processes.

The present study showed the close connection of the esterolytically active pericytic cells with the gonadotropic cells. In the control animal the most active cells were found around the FSH producing cells. After castration and during pregnancy active pericytic cells were also found near the LH cells but were not related to the thyrotropic cells during increased TSH secretion either. Some special function for this esterase activity might thus also be expected and further studies are needed for its elucidation.

The epithelial cells of the residual cleft between anterior and intermediate lobes showed an intense esterase activity preferring about the same substrates as that in the pericytic cells. The role of these cells in pituitary function is still totally unexplained. Some changes were however

noted in these cells in different experimental conditions (Ferrer 1956) The marked increase in the esterase activity in these cells after castration and adrenalectomy may probably be connected to the suggested holocrine secretion of these cells in these states The weak esterase activity in the colloid in the residual cleft may also be released from these cells It might be suggested that identical activity was found by Jirasek (1963) in certain cells lining the residual follicles in the human fetal pituitary gland

E600 resistent non specific esterases

Almost all parenchymal cell types show also an E r ns E activity but most active cells are again thyrotropic and both types of gonadotropic cells and markedly less the somatotropic cells In contrast to the E s ns E this activity is confined near the nuclei and the enzyme reaction shows coarse granular deposits In addition some diffuse background activity remains after E600 treatment in the thyrotropic and gonadotropic cells especially when 5 BIA or α NA were used as substrates At lower pH s N AS and TAA showed only the granular activity This suggests that the site of this activity may be the lysosomal particles in which very active E r ns E has been demonstrated both biochemically (Bernsohn et al 1964 Shibko and Tappel 1964) and electron microscopically (Wachstein and Meisel 1960, Torack and Barrnett 1962 Miller 1964) in liver and brain Whether similar activity present in the capillary endothelial cells is transferred from the parenchymal cells cannot be settled

Since the granular activity was significantly increased when the sections were preincubated with p CMB it may be related to the C esterase as demonstrated by Bergmann et al (1957) in hog kidney None of these activities can be cathepsin C because of their insensitivity to SH reagents or diazo salts which totally inhibit the demonstration of the enzyme using simultaneous diazo coupling (Vanha Perttula et al 1965)

The E r ns E activity seems to be very soluble, although it is suggested at least partly to be localized in the subcellular particles Lysosomal enzymes are usually quite easily solubilized e g by freezing and thawing by nonionic detergents and by hypotonic solutions (de Duve 1959) On the other hand some enzymes located in the microsomal particles are more tightly bound e g microsomal aminopeptidase in kidney tissue is rather difficult to liberate (Pfleiderer and Celliers 1963 Vanha Perttula et al 1966) although esterases seemed to be more easily solubilized as shown in the present study Differences have also been observed in the site of enzymic location in particles So acid phosphatase in lysosomes has been

claimed to be inside the particle membrane (de Duve 1959), while E r ns E is perhaps situated on the surface of the particle (Holt 1963, Shibko and Tappel 1964) from which the enzyme can be solubilized although acid phosphatase still remains inside the particles Previous studies on nervous tissue have demonstrated that E r ns Es are very soluble and cannot be visualized in fresh frozen sections (Esila 1963 Haikonen 1964, Kokko 1965 Soderholm 1965)

Also the E r ns Es are clearly connected to the functional state of the rat pituitary gland This activity was clearly augmented in gonadotropic thyrotropic, as well as somatotropic cells in states of increased secretory activity The coarse granular activity is most active in the Golgi zone and especially in the gonadotropic cells on that side of the cell facing the perisinusoidal space The Golgi apparatus is intimately connected to the release mechanism of secretory cells (Farquhar 1961) The synthetized protein is condensed to a secretory granule in the canaliculi of the Golgi zone and then migrates to the cell membrane with which it fuses The cell membrane thins out and an opening forms to the exterior and the granule is dissolved into the perivascular space The intense E r ns E in this area is probably connected to the disintegration of the lipoprotein membranes of these granules or to the production of them since in cellular metabolism the hydrolytic enzymes may be suggested to have also synthetizing properties

An interesting finding in the present studies was that after delivery this E r ns E was depleted from the gonadotropic cells The identical activity was then for some time shown in the pericytic cells which are normally devoid of all E r ns E activity This finding is in agreement with the suggested phagocytic activity of these cells The enzyme protein is extruded from the parenchymal cell and is then picked up by the histiocytic cell and gradually disintegrated This activity may correspond to the 5 BIA hydrolyzing activity in the human pituitary pericyte (Pearse 1956)

FRACTIONATION OF CARBOXYLIC ESTERASES IN THE RAT ADENOHYPOPHYSIS

LITERATURE REVIEW

Earlier fractionation studies of esterases

A method often utilized for the fractionation of enzymes in connection with histochemical investigations is electrophoresis This is usually carried out on paper or agar gel or some material such as starch gel or acrylamid having a molecular sieving effect in addition (Hunter and Markert 1957 Ornstein and Davis 1962) By these methods esterase activity can be shown by the usual histochemical reactions the counterpart for the deposition of dye in sections is thus obtained Moreover the characteristics of a multiple enzyme system can be tested in the gel slabs using various inhibitors and activators also the effect of fixatives on the enzyme can be tested Starch gel electrophoresis has been an especially favored method in the esterase studies (Hunter and Markert 1957, Markert and Hunter 1959 Hunter and Burstone 1960 Allen and Hunter 1960 Eranko et al 1962 a b 1964, Esila 1963 Harkonen 1964 Allen et al 1965 Kokko 1965 Soderholm 1965)

By electrophoresis only those esterases which are soluble (lyo enzymes) can be fractionated the firmly bound (desmo enzyme) are not demonstrable in the electrophoretic slab unless they are first solubilized by freezing and thawing or other procedures It has thus been found that the primarily soluble esterases are not demonstrable in fresh tissue sections, although they can be visualized in starch gel slabs without any solubilization procedures Lyo esterases can be separated from the desmo esterases by centrifugation of the tissue homogenates in isotonic solution Markert and Hunter (1959) showed that in mouse tissues only about 50 per cent of the total esterase activity is primarily electrophoretically mobile

Enzyme activity is not always demonstrable in a starch gel slab by the usual histochemical procedures possibly because of too weak enzyme activity low penetration of the substrate to the gel or inhibition of the enzyme in the presence of the coupling agent used To detect such enzymes the starch gel slab can be cut transversely and the enzymic activity tested in the eluates obtained after homogenization of such pieces (Vanha Perttula et al 1965)

The size of the crude enzyme samples which can be fractionated by starch gel electrophoresis is limited. Therefore other fractionation techniques must be used to obtain enzyme protein fractions from a larger tissue sample Cellulose ion exchangers provide a means for the separation of all types of proteins and has also been used for the separation of esterases (Bergmann and Rimon 1960, Svensmark 1963 Carruthers et al 1965 Vanha Perttula 1964 Vanha Perttula and Hopsu 1965a)

OWN INVESTIGATIONS

Material and methods

Substrates and enzyme assay — Substrates used in the electrophoretic and quantitative studies were commercial preparations from Sigma Chemical Comp (Ohio, U.S A.) and are listed in Table VI This table includes also the final concentration of substrates as well as the incubation times at 37 C both in the starch gel electrophoresis and in quantitative biochemical studies

In preparing the stock solutions for staining the electrophoretic slabs 0 02 mmole and for quantitative studies 0 01 mmole of the α naphthol substrates were dissolved in 3 ml of methanol the respective amount of β naphthol substrates were dissolved in 5 ml of methanol and then diluted with water up to 10 ml

The incubation medium for the starch gel slabs included

Tris HCl buffer 0 1 M pH 7 0	10 0 ml
Substrate stock solution	5 0 ml
Fast Garnet GBC solution (4 mg/ml)	3 0 ml
Distilled water or inhibitor solution	2 0 ml

The detection of 5 bromoindoxyl acetate hydrolysis in starch gel was made according to the same method as used in histochemical studies with only 0 5 mM concentration of ferro and ferricyanides

For the visualization of ChEs in starch gel slabs the Gomori s thiocholine method was applied After the incubation the slices were rinsed in several changes of hot saturated sodium sulphate solution and immersed for one minute in 1 % ammonium sulphide solution saturated with copper sulphide

Table VI Substrates used in starch gel electrophoretic as well as in quantitative enzymic studies with the final substrate concentration and incubation times at 37 C indicated

Substrate	Electrophoretic studies		Quantitative enzyme assay	
	Final substrate concentration (mM)	Incubation time (min)	Final substrate concentration	Incubation time
α naphthyl acetate	0 5	20	0 25 mM	15 min
α naphthyl propionate	0 5	20	0 25 mM	15 min
α-naphthyl butyrate	0 5	20	0 25 mM	15 min
α naphthyl valerate	0 5	20	0 25 mM	15 min
α naphthyl caprylate	0 5	60	0 25 mM	15 min
β naphthyl laurate	0 5	120	0 25 mM	60 min
β naphthyl myristate	—	—	0 25 mM	120 min
β naphthyl palmitate	—	—	0.25 mM	120 min
Naphthol AS acetate	0 5	20	0 25 mM	15 min
Naphthol AS D chloroacetate	0 5	60	—	—
Bromoindoxyl acetate	0 5	60	0 25 mM	60 min
Acetylthiocholine	1 0	12 h	0 5 mM	60 min
Butyrylthiocholine	1 0	12 h	0 5 mM	60 min

The quantitative demonstration of the enzymes was performed in the following solution

Tris HCl buffer 0 1 M pH 7 0	1 0 ml
Substrate stock solution	0 5 ml
Enzyme solution	0 5 ml

It was established that the reaction was linear with respect to time and enzyme concentration in the experimental conditions used

After incubation the liberated naphthol in the quantitative studies was coupled with 0 3 ml of aqueous Fast Garnet GBC (Edward Gurr Ltd London England 1 mg/ml) in which 10 % Tween 20 was added to disperse the dye After two minutes, 0 7 ml of 1 0 M acetate buffer of pH 4 2 was added With β naphthyl esters no Tween was added and the dye was extracted with 3 0 ml of ethyl acetate and centrifuged for 10 min at 3000 × g The absorbancies were read with a Beckman DB spectrophotometer at 540 mμ for α naphthol and β naphthol and at 545 mμ for naphthol AS derivatives Results are given in mμmole of naphthol liberated in time unit The calibration curves were made from the dilution series of α naphthol β naphthol and naphthol AS (Edward Gurr Ltd London England) initially dissolved in methanol (Vanha Perttula and Hopsu 1965a)

The hydrolysis of 5 bromoindoxyl acetate was measured by reading the intensity of the blue indigo color spectrophotometrically at 625 mμ appropriate standards were made from aqueous solutions of indigo blue (Edward Gurr Ltd., London England)

The hydrolysis of acetyl and butyrylthiocholine was determined by a modified method of Ellman et al (1961) The incubation solution was as follows

Phosphate buffer 0 1 M pH 8 0	2 0 ml
Substrate stock solution (6 mM) in water	0 25 ml
Dithiobisnitrobenzoic acid (Aldrich Chem Comp Wis U S.A 0 36 mM in 0 1 M phosphate buffer pH 7 0, in which 6 mg/10 ml NaHCO was added	0 25 ml
Enzyme solution	0 5 ml

After incubation the yellow color was measured spectrophotometrically at 412 mμ The results are given as changes in absorbancies in time unit

The affectors used in the present study were the same as previously given in Table III The modifier agent was included in the incubation medium in the appropriate concentration and half an hour s preincubation at 37 C was performed before the addition of the substrate In addition the effect of formalin and Triton X 100 on the esterase activities in starch gel slabs was tested at the final concentration of 0 05 % and 0 1 % respectively

Fractionation of pituitary esterases into bound and soluble fractions — 500 mg of fresh rat pituitary tissue was homogenized in 50 ml of 0 25 M sucrose solution at +4 C Ten ml of this solution was diluted to 200 ml with water and used as the *total homogenate* stock solution The remaining 40 ml of the homogenate was adjusted to pH 5 0 with 0 1 M HCl after standing for one hour the sample was centrifuged for 60 min at 15 000 rpm in an International high speed refrigerated centrifuge model HR 1 (International Equipment Co Mass U S A) The supernatant fraction (*pH 5 supernatant*) was neutralized and diluted with water up to 400 ml The sediment was suspended in 40 ml of 0 25 M sucrose and half of this fraction was diluted to 200 ml with water

(*pH 5 sediment*) To the other half, 0 05 % Triton X 100 was added After standing 60 min, this fraction was centrifuged as reported earlier Both the supernatant and sediment fractions were diluted to 100 ml (*Triton supernatant* and *Triton sediment* fractions) with water after centrifugation

Starch gel electrophoresis — The technique used in the starch gel electrophoresis was in principle the same as that of Markert and Hunter (1959) For this study the anterior pituitary tissue was frozen at —40 C A sample of 100 mg of tissue was homogenized in 0 3 ml of distilled water with a teflon pestle glass homogenizer (Thomas Philadelphia, U S A) The homogenate was frozen and thawed five times and centrifuged for 10 min at 20 000 × g One tenth ml of the supernatant solution was pipetted on to stripes of Whatman No 1 filter paper (8×0 6 cm) A piece of such paper was inserted into a slit made with a razor blade in the gel In preparing the starch gel 36 g of hydrolyzed starch (Connaught, Toronto Canada) was mixed and boiled with 300 ml of 0 076 M Tris HCl buffer pH 8 65, and poured into a plastic tray (dimensions 25 × 8 × 0 6 cm) A transverse slit was cut 5 cm from the cathodal end of the tray The bridge buffer used in both sides was 0 3 M boric acid NaOH buffer, pH 8 0 eight filter paper sheets connected the buffers to both ends of the gel tray

Electrophoresis was carried out for 4 hours with a voltage gradient of 10 volts/cm using an LKB electrophoresis power supply (LKB Produkter, Stockholm Sweden) During the electrophoresis the gel was covered with a plastic foil to avoid evaporation and cold water was running beneath the gel to keep the temperature near +4 C During the electrophoresis the anodal front migrated 15 cm After electrophoresis the starch plate was sliced longitudinally into ten slab each 0 8 cm broad After histochemical reaction the slabs were rinsed in water and stored in glycerol

DEAE cellulose column chromatography — The techniques used for esterase chromatography have been reported in a separate communication (Vanha Perttula and Hopsu 1965a)

Results

Studies with tissue homogenate

In the present studies of a homogenate and its fractions after centrifugation and treatment with Triton X 100 the hydrolytic activities were tested in 0 1 M Tris HCl buffer of pH 7 0 regardless of the preferred pH area for the individual substrates earlier described by Vanha Perttula and Hopsu (1965a) The results are presented in Table VII It was found that during the fractionation procedures the hydrolytic activities increased differently with respect to different substrates The hydrolytic activities of the Triton fractions and the pH 5 supernatant were pooled and the percentage increase was calculated for the different substrates as follows α NA 75 5 % α NB 122 6 % N AS 13 6 %, 5 BIA 19 4 %, and β NP 42 6 %

The esterases hydrolyzing α NA α NB, and β NP seem to be particle bound to a great extent whereas N AS and 5 BIA are hydrolyzed better

5

Table VII Hydrolysis of different e ter substrates by the pituitary homogenate, the soluble and particle fractions and by the fractions after the extraction of the bound esterases by Triton X 100 Also shown are the percentage changes in the hydrolytic rates after some modifier substances

Fraction	Hydrolysis rate (mμmole/min)	E600 10 μM	E erine 10 μM	NaF 1 mM	p CMB 0 1 mM	TC 1 mM	Perazine 1 mM	Cu 0 1 mM
Substrate α naphthyl acetate								
Total homogenate	2 589	—84	0	—56	0	—51	—22	—20
pH 5 supernatant	1 726	—73	0	—67	0	—37	—30	—17
pH 5 sediment	2 255	—87	—3	—64	—3	—46	—17	—24
Triton supernatant	1 151	—89	—6	—54	—8	—77	—24	—40
Triton sediment	1 668	—86	—3	—72	—6	—34	—27	—22
Substrate α naphthyl butyrate								
Total homogenate	4 257	—97	0	—91	0	—19	—24	—10
pH 5 supernatant	3 970	—99	0	—90	0	—28	—18	0
pH 5 sediment	4 200	—99	0	—89	—8	—14	—14	0
Triton supernatant	2 158	—99	—3	—98	—18	—40	—25	—6
Triton sediment	3 348	—98	—1	—90	0	—7	—18	0
Substrate naphthol AS acetate								
Total homogenate	0 921	—6	0	—2	+31	—12	0	—47
pH 5 supernatant	0 652	—2	0	0	+40	—2	0	—13
pH 5 sediment	0 342	—7	—2	—7	+14	—14	0	—29
Triton supernatant	0 223	—10	0	0	+10	—39	0	—27
Triton sediment	0 171	—25	0	—2	—16	0	0	—24

Table VII (continued)

Fraction	Hydrolysis rate (mμmole/10 min)	E600 10 μM	Eserine 10 μM	NaF 1 mM	p CMB 0 1 mM	TC 1 mM	Perazine 1 mM	Cu 0 1 mM
Substrate 5 bromoindoxyl acetate								
Total homogenate	3 389	0	0	—29	+16	—36	—	—93
pH 5 supernatant	1 920	0	0	[illegible]	+16	—27	—	—98
pH 5 sediment	1 570	—3	—5	—53	—6	—34	—	—79
Triton supernatant	1 403	—47	—17	—73	—45	—20	—	—98
Triton sediment	0 722	—25	—13	—50	0	—26	—	—84
Substrate β naphthyl palmitate								
Total homogenate	0 598	—45	0	0	—[illegible]4	—86	—79	—78
pH 5 supernatant	0 136	—88	0	—1[illegible]	—63	—32	—39	—74
pH 5 sediment	0 347	—43	0	—14	—93	—95	—99	—99
Triton supernatant	0 239	—15	0	—15	—77	—9[illegible]	—99	—94
Triton sediment	0 478	—25	0	—5	—[illegible]9	—84	—99	—98

by the soluble fraction Triton X 100 solubilized about 40 % of the enzymes hydrolyzing α NA and α NB 33 % of the enzymes hydrolyzing β NP, and about 60 % of the enzymes hydrolyzing N AS and 5 BIA from the pH 5 sediment fraction

The enzymes hydrolyzing α NA and α NB and also those hydrolyzing β NP are readily inhibited by E600 the two former also are markedly inhibited by NaF Only the enzymes liberated from the particles were sensitive to E600 when N AS and 5 BIA were used as substrates The contribution of ChEs to the hydrolysis of these substrates seems to be slight p CMB seems to inhibit markedly the hydrolysis of β NP, but has a significant activating effect on the hydrolysis of N AS and 5 BIA while the hydrolysis of α NA and α NB is only slightly modified Sodium tauro cholate (TC) as well as perazine inhibited moderately the hydrolysis of α NA and α NB but they markedly inhibited that of β NP The hydrolysis of N AS was not modified by perazine (turbidity prevented the measure ment of the hydrolysis of 5 BIA)

Cu^{++} almost totally inhibited the hydrolysis of 5 BIA and diminished also markedly the hydrolysis of β NP and N AS α NB was almost unaf fected The discrepance between the reaction of N AS and 5 BIA to Cu^{++} inhibition was somewhat surprising since these substrates had otherwise analogous reactions It was observed, however, that Cu^{+} in biochemical enzyme studies can inhibit the blue indigo color formation and this can be prevented by adding ferro and ferricyanide in 0 5 mM concentration to the incubation medium as is done in the histochemical method

Starch gel electrophoresis

Histochemical staining of the starch gel slabs

Thiocholine esters — By the present method it was not possible to localize the activities which hydrolyze AcThC and BuThC even when using the long incubation times as in the histochemical method

α Naphthyl acetate — This substrate produced the largest number of bands (Fig 70 Table VIII) Altogether 11 positive bands were iden tified which are designed 1—11 and counted from the anodal end to the origin (Webb 1965) No bands were obtained on the cathodal side with any of the substrates used under these electrophoretic conditions The most active bands were 2 3 4 and 7—11, of which bands 7—11 were near each other Bands 10 and 11 remained after incubation with E600 (Fig 70) Other E600 resistent activity bands were 2 and 3 The others

were clearly inhibited also by NaF. DFP inhibited markedly all enzymic activities. Cu^{++} inhibits moderately activity zones 10 and 11 and totally band 3. Formalin treatment totally inhibits bands 7—9 which are also somewhat inhibited by Triton X 100 in 0.1 % concentration. Bands 10 and 11 are somewhat activated by p-CMB and band 4 by Triton X 100.

α-Naphthyl propionate.—The most apparent difference between α-NPr and α-NA was the weakness of the bands 10 and 11 (Fig. 70) which after E600 treatment were only slightly visible. Without E600 this activity was for the most part covered by very intense activity of bands 7—9. Also the E r ns E bands 2 and 3 were clearly decreased in activity. On the other hand, activity bands 5 and 6 as well as 1 were clearly more active. However, the formalin-sensitive bands 7—9 (Fig. 70) and band 4 were the most active bands obtained.

α-Naphthyl butyrate. — Bands 3, 10 and 11 were absent with α-NB (Fig. 70). Bands 1, 5 and 6 were increased in activity but on the whole bands number 4, 8 and 9 were most active. Band 2 and 7 were quite weak. All these activity bands except band 2 were totally inhibited by E600 (Fig. 70) and NaF. Bands 5 and 6 were best visualized with this substrate. It was found that when the homogenate was made only of the lateral parts of the pituitary gland this activity could not be found. Bands 5 and 6 were most active when starch gel was carried out with a homogenate of the cleft epithelium area of the pituitary gland (Fig. 70) or with a homogenate of the intermediate lobe. This suggests that these two activity zones were derived from the cleft epithelial activity. The characteristics of this activity are clearly reminiscent of those in the histochemical study.

α-Naphthyl valerate, α-naphthyl caprylate and β-naphthyl laurate. — With α-NV (Fig. 70) the same activity bands were obtained as with α-NB although they were much weaker. The decrease was most apparent in bands 7—9, however band 7 was never seen with certainty. With α-NC only bands 1 and 4 appeared (Fig. 70). Both these activities were inhibited by taurocholate. With β-NL no activity could be produced by the usual method or by preincubating the starch gel slab without Fast Garnet GBC and making a postcoupling reaction.

Naphthol AS acetate and naphthol AS-D chloroacetate. — Zymograms produced with these substrates showed somewhat different band patterns. N-AS (Fig. 70) was hydrolyzed most strongly by E600-resistent bands 10 and 11. Bands 2 and 3 were also clearly positive. The only E600-sensitive activity was band 4, which showed slight activity with this substrate. The enzyme activities revealed by N-AS-D (Fig. 70) were much weaker than those of N-AS. This substrate showed that band 2 was the most active, but a slight activity was found also in bands 4, 10 and 11.

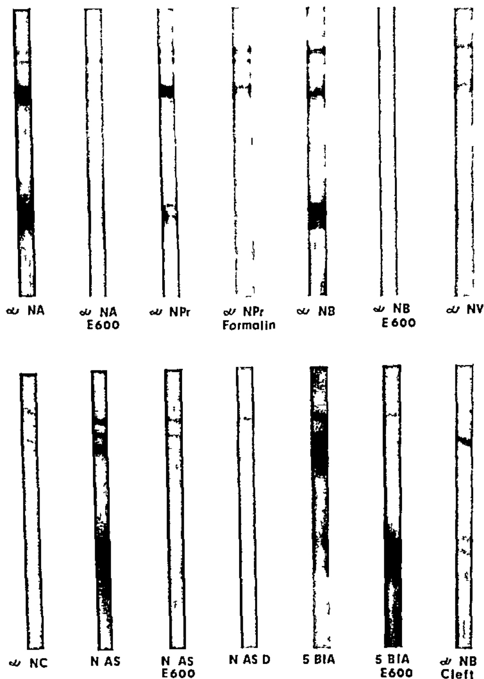


Fig 70 Esterases in zymograms obtained with different substrates and inhibitors Incubation times for the different substrates are given in Table VI

5 Bromoindoxyl acetate and thioacetic acid — Zymograms produced with 5 BIA using a modified indoxyl esterase method in which the ferro ferricyanide redox buffer was reduced to 10 per cent (0 5 mM) of the original histochemical method gave a positive enzyme reaction in bands 2, 3, 4 10 and 11 (Fig 70) The relative intensities of these bands varied in relation to those obtained with N AS Bands 3 and 4 were clearly more active Only band 4 was inhibited by E600 (Fig 70) Triton X 100 or formalin had no effect but p CMB had some activating influence on bands 10 and 11 Identical activity bands were obtained using TAA Eserine was without effect with all substrates tested in starch gel electrophoresis This shows the minimal contribution of ChEs in the enzymic composition of rat pituitary gland

Substrate preference of the different activity bands (Table VIII) — Starch gel bands 10 and 11 were always demonstrated by the same substrates at about equal intensity They may be called acetylesterases because they were preferentially revealed by N AS and well demonstrated also by α NA 5 BIA and TAA Weak activity was found with N AS D and α NPr This enzyme was resistent to E600 but p CMB had a clearly activating effect These are the characteristics of C esterase as presented by Bergmann et al (1957) and Bergmann and Rimon (1958 1960) The intensity of the hydrolysis of TAA can not be correlated because of different conditions in the incubation medium

Band 7—9 were best visualized with α NPr This was followed with about equal intensity by α NA and α NB Band 7 was however clearly weaker with α NB than with α NA This band was always absent with α NV but bands 8 and 9 could be clearly shown α NC N AS, and 5 BIA showed no activity in these bands or the activities may be hidden by bands 10 and 11

Bands 5 and 6 have equal enzymatic characteristics for substrate specificity and inhibitors The most representative substrate was α NB Both shorter and longer chained naphthol substrates were hydrolyzed with decreasing intensity

Band 4 represents an E s ns E activity and can be revealed about equally well with α NA α NPr and α NB The hydrolytic activity with α NV and α NC was clearly smaller but it was, however, the most active band This band was also shown by N AS N AS D and 5 BIA This band represents the only E s ns E activity obtained with these substrates

Band 3 was obtained with all acetate esters and only a very weak activity was found with α NPr This enzyme thus represents the same kind of acetylesterase activity as enzymes in bands 10 and 11 The relative

substrate specificity between different acetate esters however is different to some extent The most preferred substrates were α NA and 5 BIA, while N AS gave clearly weaker intensity in this band

Band 2 also hydrolyzed preferentially acetate esters but a clear hydrolytic activity was found also using other ester substrates as long as α NV With α NC the reaction was negative This band represents the only E i ns E found with α NB or longer chained substrates This enzymic band somewhat preferred N AS in relation to 5 BIA while the contrary was true in band 4

Band 1 contained the most mobile enzymic activity The preferred substrate was α NB although α NPr seems to be hydrolyzed at about an equal rate α NA and α NV are weaker substrates and with α NC only minimal activity could be revealed This enzyme activity was sensitive to E600 and NaF

Bound and soluble esterases — When starch gel electrophoresis was carried out using the supernatant of the pituitary homogenate and correlated with that obtained with whole tissue homogenate it was evident that some esterase bands showed clearly decreased enzymic activity The most apparent decrease was found in the bands 7—9 which thus seem to represent particle bound esterase activities All the other activities are apparently more easily solubilized although they may in tissue be bound to some particles When the sediment fraction was solubilized by repeated freezing and thawing after which electrophoresis was carried out, the most active bands were 7 to 11 This demonstrates that also bands 10 and 11 may be largely particle bound but easily released into solution

Bands 1—6 are found in the electrophoretic pattern of the supernatant fraction and are thus clearly soluble or lyo enzymes

Studies with starch gel eluates

When starch gel slabs were cut transversely and the activity hydrolyzing AcThC and BuThC determined from the eluate two stronger hydrolytic areas were obtained with both of the substrates (Fig 71) The former had its activity maximum in sections 5—6 which was not coincident with any of the previously reported activity bands in starch gel slabs This activity was resistent to eserine and E600 The second activity had its maximum in sections 11—12 thus corresponding to the area of the esterase bands 7—11 This activity was totally inhibited with eserine while the bands corresponding these fractions were resistent to it Some slight inconsistent hydrolysis of the thiocholine esters was observed also in sections further toward the anodal side

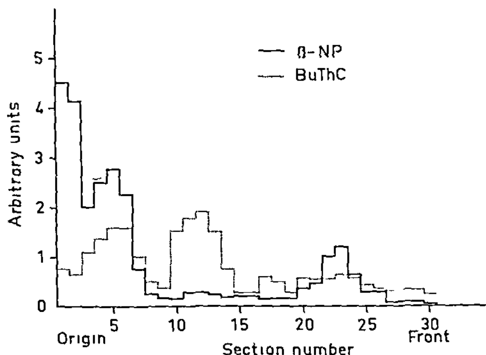


Fig 71 Distribution of the hydrolytic activities toward β NP and BuThC after electrophoretic separation of the pituitary homogenate in starch gel.

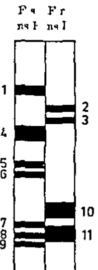
The hydrolysis of β NL β NM and β NP was also tested with the eluates (Fig 71) It was found that all these substrates were markedly hydrolyzed by the very first sections which usually showed much weaker activity against the short chained fatty acid esters This activity was markedly inhibited by I 600 taurocholate and perazine The second activity area was coincident with the first activity against thiocholine esters This activity was not inhibited by E600 but some inhibition was obtained with taurocholate and perazine A third stronger activity was observed in sections 22—23 which corresponded to the area of the 4th activity band in starch gel This activity was most prominent with β NL of the long chained ester substrates

Correlation of esterases in sections and zymograms (Table VIII)

The strong E i ns E activities in gonadotropic thyrotropic and somatotropic cells most likely correspond to the activity bands 10 and 11 in zymograms These two bands may represent different cellular origins, which however cannot be demonstrated by substrate specificity or in

VIII Schematic zymogram illustrating the results obtained by starch gel electrophoresis with different substrates and offering the suggestion
e probable correlation with the site of activity in histochemical studies

nd	α NA	α NPr	α NB	α NV	α NC	N AS	N AS D	5 BIA	TAA	Site of activity in pituitary tissue
1	++	+++	+++	++	±	—	—	—	—	pericytic cells (?)
2	+++	++	+	+	—	++	+	++	+	diffuse activity in fixed TSH & LH cells
3	++	+	—	—	—	+	—	++	—	capillary endothelium
4	+++	+++	+++	++	+	++	+	++	+	pericytic cells
5	+	++	+++	++	—	—	—	—	—	cleft epithelium
6	+	++	+++	++	—	—	—	—	—	
7	++	+++	+	—	—	—	—	±	—	activity in different cell types in unfixed sections
8	++	+++	++	+	—	—	—	±	—	,
9	++	+++	++	+	—	—	—	±	—	
0	++	+	—	—	—	+++	+	+++	++	granular activity in fixed FSH LH & TSH cells
1	++	+	—	—	—	+++	+	+++	++	,



inhibitor studies, such studies do not differentiate these enzyme bands. Both these bands were clearly stronger after castration which has the most effective action on the granular E.-i.ns.E. in fixed sections.

Bands 7—9 were almost totally inhibited by formalin which in histochemical reactions was found to inhibit the E.-s.ns.Es in unfixed sections. These three bands may thus be derived from various cell types which have the same type of enzymic activity tested in fresh sections. Band 7 was weaker than bands 8 and 9 when α-NB was used as substrate and was found no more with α-NV. This may suggest somewhat different characteristics of this enzyme in different cellular locations. After castration bands 8 and 9 were clearly much increased; these bands may represent the activity found in LH and FSH gonadotropic cells although the identical activity in the thyrotropic cells may contribute. The 7th band may then represent the activity in the somatotropic cells. Triton X-100 (0.05 %) gave a slight inhibition of bands 7—9 when α-NPr was used as substrate.

Bands 5 and 6 were not present in starch gel when only the lateral part of the pituitary gland was taken for the homogenate but they were very strong in preparations in which also the cleft epithelial tissue was included. A strong enzyme activity at the same level was obtained when starch gel was carried out using intermediate lobe homogenate. The substrate specificity of these bands clearly correspond to those obtained in histochemical studies on cleft epithelium.

Band 4 had the largest substrate spectrum and was E 600-sensitive. It was not inhibited by formalin treatment; on the contrary it was somewhat activated by Triton X-100, which was always more marked when the chain lenght of the substrate increased. The same effect was found in the pericytic enzyme activity. This enzyme activity was also very much increased after castration but no increase was found after methylthiouracil tratment; this effect was found in histochemical studies too.

Band 2 and 3 are probably different enzymes on the basis of the difference in substrate specificity. Band 3 was best revealed by α-NA and 5-BIA; these are the substrates which show the greatest diffuse activity which was localized in capillary endothelial cells in formalin fixed sections. The diffuse E.-r.ns.E in the FSH- and LH-cells was visualized best by acetate esters but some weak activity can be shown with other substrates too and may correspond to band 2 activity. Such an activity was also obtained with NAS-D.

Band 1 was very sensitive to E600 but was unaffected by formalin. The corresponding histological site is at present unclear. This band was not clearly increased in activity in relation to other activities after castration.

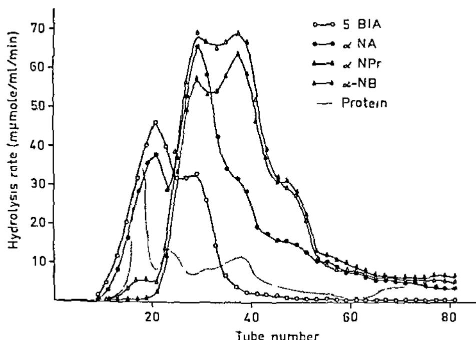


Fig 7* Separation of the enzymic activities hydrolyzing different estera e sub trates on a DFAE cellulose hromatography fractionation at pH 8 2 Incubation times for dif ferent substrates are given in Table VI

DFAE cellulose column chromatography

This part of the study has been the subject for a separate communication (Vanha Perttula and Hopsu 1965a), where the esterase fractions at pH 7 0 are described in detail Therefore only those results will be described here which were obtained by chromatography at pH 8 2 and which differ es sentially from those at lower pH

The distribution of the hydrolysis of ester substrates chromatographed at pH 8 2 is given in Figs 72 and 73 As a rule poorer separation of the different activity peaks was obtained in comparison to the chromatography at pH 7 0 However at pH 8 2 the first E s ns E peak could be divided into two parts with different substrate specificities α NA was best hyd rolyzed in the former part of the peal (Fig 72 tube 29) and showed clearly weak activity in the later tubes where however marl ed hydrolysis of α NPr α NB α NV and α NC (Fig 73 tube 39) occurred. No differen ces in the inhibitor characteristics in the different parts of the peal could be detected

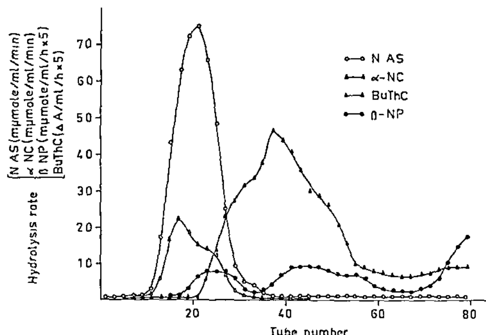


Fig 73 Separation of the enzymic activities hydrolyzing N AS α NC BuThC and β NP on a DEAE cellulose chromatography fractionation at pH 8 2 Incubation times for the substrates are given in Table VI

The fourth activity peak was most active in the hydrolysis of long chained fatty acid esters and it was not totally eluted out of the column by the 0 35 M NaCl concentration at pH 8 2 (Fig 73 tube 80) The hydrolytic activities against thiocholine esters were not favorably separated from the ns F peak in the pH 8 2 chromatography

Discussion

The present fractionation study of rat pituitary homogenate by chromatography and electrophoresis shows the presence of a variety of esterolytic activities Most of the esterase fractions differ from each other in respect to substrate specificity modifier characteristics and pH optima, this makes it possible to estimate their activity separately even in a non fractionated pituitary However there are different variants of both E s ns E and E r ns F activities which cannot be separately analysed

The four E r ns F bands in starch gel electrophoresis were not separable in chromatographic fractionation This activity represents a typical acetyl

esterase activity (Hofstee 1960) with decreasing substrate specificity order of N AS, 5 BIA, α NA and α NPr The E 1 ns E runs in the electrophoretic fractions labelled by numbers 10 and 11 in this study but a minor portion of it forms also the fractions 2 and 3 The latter differ somewhat in their substrate preferrence from the activities in bands 10 and 11 These enzyme activities may represent either isozymes or may be derived from different cell types producing nearly identical enzymes in other characteristics except for a slight difference in electrophoretic mobility In histochemical studies this type of activity was present in formalin fixed sections in different cells, mainly however in gonadotropic thyrotropic, and somatotropic cells

The term isozymes was primarily introduced to describe an electrophoretically separable group of enzymes with otherwise identical enzymatic characteristics (Markert and Møller 1959) This enzymic heterogeneity may be due to the occurrence of a truly different species of native proteins of precursors or degradation products or of proteins modified *in vivo* or in the preparatory procedure In human serum and different tissues a group of electrophoretically and chromatographically separate ns ChEs have been demonstrated which differ from each other with respect to their sialic acid content without any difference in other molecular properties (Svensmark 1965) In a recent study Cann (1966) has demonstrated that a purified protein molecule (bovine serum albumin) in electrophoresis interacts reversibly with borate buffers resulting in multiple protein zones which however do not indicate the presence of altered protein structure In the present study the electrodic buffers also contained borate and it is probable that e g bands 10 and 11 may represent a single enzymic species which during the electrophoretic separation has reacted reversibly with the buffer ions

The possible identity of E r ns E with other known enzymes has been much debated Originally it was suggested on the basis of histochemical studies of Pepler and Pearse (1957) and Hess and Pearse (1958) that the enzymic activity hydrolyzing 5 BIA may be cathepsin C because this proteolytic enzyme had been shown to exhibit esterolytic activity against substrates *like glycyl phenylalanyl glycyl tyrosyl and prolyl phenylalanyl* ethyl esters (Wiggans et al 1954) Studies with preparation of hog kidney (Vanha Perttula et al 1965) however showed that cathepsin C is separate from E r ns Es Observations using a new cathepsin C substrate glycyl phenylalanyl β naphthylamide have demonstrated that rat pituitary tissue has minimal or no hydrolytic activity toward this substrate at the pH range of cathepsin C although it was used in the presence of cysteine and

EDTA which are known to activate this enzyme (Vanha-Perttula, unpublished observations).

It has also been suggested (Holt 1963) that another proteolytic enzyme, cathepsin D, may be involved. This enzyme hydrolyzed hemoglobin at acid pH (Press et al. 1960). When the hydrolysis of hemoglobin was tested in fractions of DEAE-cellulose chromatography at pH 3.8, no significant hydrolytic activity coincided with the E.s.ns.E. peak (Vanha-Perttula and Hopsu 1965a). The esterase peaks were neither coincident with the activities hydrolyzing leucyl-β-naphthylamide (Vanha-Perttula and Hopsu 1965b). In pituitary homogenates the hydrolysis of N-AS and 5-BIA is somewhat activated by low concentrations of organic mercurials, e.g. p-CMB, but after further purification this activation is lost. These characteristics as well as the pH optimum for the hydrolysis are the same as those presented for a new esterase type called C-esterase by Bergmann et al. (1957) and Bergmann and Rimon (1958, 1960).

In starch gel electrophoresis 7 different E.s.ns.E. bands were obtained. They could be partially correlated with the histochemical localization on the basis of substrate specificity and differences in experimental conditions. Three enzymes which migrated the shortest distance in the electrophoresis were found to be identical with the desmo-enzymes which were responsible for the histochemical reaction in unfixed section.

Although there are different enzyme bands in starch gel electrophoresis which are similar in effector studies, this does not mean that the enzymes can be termed as isozymes. As in the present investigation there is reason to suggest that enzymes of similar modifier characteristics have different cellular locations and slightly different substrate preferences. With suitable modifier substances these may be divided into clearly different subgroups. E.s.ns.E. bands 5 and 6 as well as 7—9 have nearly identical characteristics with respect to substrate specificity. The former correspond clearly to the E.s.ns.E. activity in the cleft epithelial cells and the three latter bands which were sensitive to formalin treatment are the counterpart for the fine granular cytoplasmic activity in gonadotropic, thyrotropic, and somatotropic cells. The question arises whether they are really derived from the different cell types or represent only an enzymic heterogeneity which is produced by the borate buffer during the electrophoresis (Cann 1966). In any case the four activity groups (1—4, 5—6 as well as 7—9) can be taken as separate E.s.ns.E.s which also have clearly different locations in the rat anterior pituitary cells and after fractionation by DEAE-cellulose chromatography.

The first E.s.ns.E. peak in pH 8.2 chromatography preferred α-NPr as

substrate, this was also the best substrate for the bands 7—9 The second half of this peak which was more evident in the chromatography at pH 8 2 than at pH 7 0 (Vanha Perttula and Hopsu 1965a), hydrolyzed α NB and α NV about equally as well as α NPr while α NA seems to be much inferior as a substrate This peak most probably corresponds to activity band 4 in starch gel electrophoresis and the pericytic activity in the histochemical reaction The esterolytic activity in the cleft epithelial cells preferred the same substrates as the pericytic activity In chromatography at pH 7 0 a small separate E s ns E peak was found (Peak III Vanha Perttula and Hopsu 1965a), in the pH 8 2 chromatography it is only partially separate from the earlier peak This peak has the same substrate specificity in addition to identical affector characteristics as the electrophoretic bands 5 and 6

Band 1 differed from the other E s ns Es in substrate specificity α NPr and α NB were the preferred substrates and no hydrolysis of N AS N AS-D and 5 BIA occurred This activity was weaker than the other E s ns E bands and its counterpart in the chromatography may hide behind the other peaks Its histochemical localization is also uncertain It might be a second enzyme species in the pericytic cells or correspond to the weak parenchymal E s ns E activity in fixed gonadotropic cells

The demonstration of AChE and ns ChE activities in the histochemical work using AcThC and BuThC as substrates has been the preferred method using Gomori s modification (1952) of Koelle s method (1951) Indoxyl (Adams 1949 Holt 1952) and naphthol esters (Pepler and Pearse 1957 Pearse 1961) have also been used for the demonstration of ChEs and differentiated from the ns Es by specific inhibitors The ns ChE activity in the rat pituitary thyrotropic cells was so weak that its contribution to the hydrolysis of these latter substrates could not be revealed In quantitative homogenate studies with α NA as substrate the inhibition of the hydrolysis was minimal by eserine in starch gel electrophoresis no eserine sensitive band could be found Quantitative studies using the method of Ellman et al (1961) showed that the ns ChE peak in chromatography was totally separate from all activity hydrolyzing naphthol and indoxyl esters (Vanha Perttula and Hopsu 1965a) Barron et al (1966) have also found that the feline muscle ChEs are histochemically demonstrable with α NA but in quantitative studies their contribution is minimal A preparation of human plasma cholinesterase has been shown to hydrolyze α NA in a biochemical system (Hopsu and Pontinen 1964) These discrepancies between biochemical and histochemical results cannot be solved without further studies

A peculiar eserine and E600-resistent enzymic activity hydrolyzing thiocholine esters was found histochemically in or near the capillary endothelial cells. This activity was also found in the eluates of the starch gel as well as after DEAE cellulose chromatography as a separate peak. It was, however, not separable from an activity hydrolyzing long-chain naphthol esters and trifluoroacetyl α-naphthylamide (Vanha-Perttula and Hopsu 1965a). This may correspond to the eserine-resistent activity in the rat hypothalamic blood vessels which hydrolyzed the same substrates but did not split 5-BIA (Holmes 1961).

The histochemical literature is full of observations of enzymic activities in different tissues and different cell types. Efforts have been made to study, e.g., tissue esterases both histochemically and after electrophoretic separation, but an approach for the correlation between the results has remained inadequate (Eskelä 1963, Fränkö et al. 1964, Harkonen 1965, Kokko 1965, Söderholm 1965). The present work shows that a large series of substrates and inhibitors as well as more than one fractionation method is required in order to get data enough for an appropriate correlation.

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Band 1 differed from the other E's ns E's in substrate specificity. α-NPr and α-NB were the preferred substrates and no hydrolysis of N-AS-NASD and 5-BIA occurred. This activity was weaker than the other E's ns E bands and its counterpart in the chromatography may hide behind the other peaks. Its histochemical localization is also uncertain. It might be a second enzyme species in the pericytic cells or correspond to the weak parenchymal E's ns E activity in fixed gonadotropic cell.

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QUANTITATIVE ESTIMATION OF CARBOXYLIC ESTERASES IN EXPERIMENTAL CONDITIONS

In the histochemical studies marked changes were observed in the enzymic reactions in different cells under different experimental conditions. Many factors may contribute to these changes. In fresh frozen sections the readily soluble E r ns Es were not demonstrated at all and in fixed tissue the E s ns E in gonadotropic and thyrotropic cells was almost totally inhibited by formalin. In addition the diazo salts may inhibit different enzymes to varying degrees. In order to avoid these drawbacks esterase activity was estimated quantitatively from the pituitary homogenates. On the basis of the performed histochemical and fractionation studies four different substrates were chosen to be used in the quantitative studies: α NA which is hydrolyzed by a large number of esterase components, NAS which is the preferred substrate for the E r ns Es, α NB which is hydrolyzed only slightly by E r ns Es and demonstrates thus mainly E s ns Es, β NP which is mainly hydrolyzed by esterases having substrate preference to long chained naphthol esters.

OWN INVESTIGATIONS

Material and methods

The material consisted of 326 albino rats of the Sprague Dawley strain which were bred at the estate of the Pharmacy Orion at Mankkaa. Of them 196 were males and 130 were females; they were about 4 months of age at the beginning of the experiment. The animals were divided into 29 groups which were separately housed in cages, however the control groups were in six different cages. The number of animals and the treatment given are presented in Table IX. Three subgroups received the same treatment but the time of treatment was different: group I for 10 days, group II for 20 days and group III for 30 days. All animals were fed *ad libitum* and fresh water was supplied each day for all groups except the adrenalectomized rats which received 0.9 per cent saline solution to drink. Table IX also gives the initial and final mean weights for each group.

The animals were killed by decapitation and some endocrine organ weights were measured. The pituitary gland was removed immediately, weighed first as a whole and again after removal of the posterior and intermediate lobes with a pin. After this each anterior pituitary gland was homogenized in 10 ml of distilled water. These samples were then stored in a refrigerator at +4 C.

The substrates used were α NA, α NB, NAS and β NP. E600 (10 M) was included in the incubation medium for NAS hydrolysis. The hydrolysis rates of the

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Material and methods

The material consisted of 326 albino rats of the Sprague Dawley strain which were bred at the estate of the Pharmacy Orion at Mankkaa Of them 196 were males and 130 were females they were about 4 months of age at the beginning of the experiment The animals were divided into _9 groups which were separately housed in cages how ever the control groups were in six different cages The number of animals and the treatment given *are presented in Table IX Three subgroups received the same treat* ment, but the time of treatment was different group I for 10 days group II for 0 days and group III for 30 days All animals were fe l *ad libitum* and fre h water was supplied each day for all groups except the adrenalectomized rats which received 0 9 per cent saline solution to drink Table IX also gives the initial and final mean weights for each group

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The substrates u ed were α NA α NB N AS and β NP E600 (10 M) was in cluded in the incubation medium for N AS hydrolysis The hydrolysis rates of the

Table IX Initial and final body weights and the weights of the anterior pituitary gla

Experimental condition	Sex	No of animals	Duration of treatment (days)	Body
				Initial
Ol arach 0 1 ml s c	male	24	20	203 ± 1° 4
Thyranon 0 2 % in food	male	10	10	210 ± 1°.5
, 0 2 % in food	male	10	20	211 ± 8 9
„ 0 2 % in food	male	11	30	194 ± 8 9
MTU ‿0 % in food	male	8	10	233 ± 8 0
, 2 0 % in food	male	10	20	° 8 ± 4 9
„ 2 0 % in food	male	10	30	°°7 ± 11 1
Neo Hombreol 1 0 mg s c	male	9	10	230 ± 1 6
„ 1 0 mg s c	male	10	20	219 ± 5 5
„ 1 0 mg s c	male	10	30	186 ± 3 7
Castration	male	10	10	23? ± 9 5
,	male	9	20	213 ± 10 0
	male	12	30	194 ± 6 4
Adreson 1 0 mg s c	male	8	10	2°5 ± 7.2
„ 1 0 mg s c	male	10	20	°39 ± 8 4
1 0 mg s c	male	10	30	187 ± 6 2
Adrenalectomy	male	10	10	°35 ± 4 9
,	male	8	20	234 ± 7 7
„	male	7	30	219 ± 8 6
Ol arach 0 1 ml s c	female	°2	20	189 ± 7 4
Progestin 1 0 mg s c	female	12	10	°09 ± 4 9
1 0 mg s c	female	12	20	210 ± 4 8
1 0 mg s c	female	12	30	206 ± 10 1
Dimenformon 0 1 mg s c	female	11	10	‿15 ± 7 6
, 0 1 mg s c	female	11	20	2°1 ± 4 5
„ 0 1 mg s c	female	13	30	197 ± 6 7
Castration	female	11	10	°13 ± ‿4
,	female	13	20	233 ± 7 9
	female	13	30	‿10 ± 4°

Standard deviation
$p < 0\,05$
[b] $p < 0\,01$ } Compared with controls
$p < 0\,001$

substrates were measured as described in the preceding chapter Details of the sub strate concentrations and incul ation times are given in Table VI Duplicate enzyme assays were made with each substrate Duplicate d terminations of the protein content of the enzyme samples were made by a modified method for amide nitrogen (Stegemann 1958) in which the color after nesslerization was stabilized with KCN (Minari and Zil versmit 1963) Each series of determinations was followed by appropriate standards of ammonium sulphate

adrenal, testes or ovaries as well as the thyroid gland in the experimental serie

weight (g)	Anterior pituitary gland (mg)	Adrenals (mg)	Testes vs ovaries (mg)	Thyroid gland (mg)
Final				
212±10 5	5 7± 0 9	49 6±10 1	2998±183	9 1±1 8
195± 9 6	4 8± 0 9[b]	46 8± 5 8	3°88±118	8 1±2 2
186± 8 5	5 0± 0 6	50 1± 4 8	3157±1°0	7 4±1 8[b]
168± 7 4	4 7± 0 8[b]	54 1± 7 5	3069±197	6 9±1 4
249± 7 9	6 9± 1 0[b]	46 5± 5 1	3709±165[b]	33 5±5 3
235± 6 5	8 0± 1 1	59 0± 4 8	3335±134	35 2±7 2
237±12 3	9 5± 0 7	36 8± 4 4	3331±126	4°0±6 1
°35± 9 4	5 1± 0 7	41 0± 9 8[b]	2608±118[b]	
223± 4 8	4 9± 1 0	41 3± 4 3[b]	2552± 76	
20-± 5 3	4 5± 0 5	37 7± 8 0	2363± 55	
210± 7 7	6 3± 0 9	43 4± 6 6[b]	—	
205± 8 6	6 7± 0 5[b]	46 1± 4 5	—	
196± 7 3	7 0± 1 0	40 1± 5 5[b]	—	
-10± 7 5	5 6± 0 6	33 9± 3 2	2796±168	
°05± 6 9	6 7± 0 7[b]	3° 4± 3 1	2519±127	
164± 6 3	5 5± 0 8	23 0± 6 6	2435±106	
-14± 6 8	6 7± 1 2	—	2940± 78	
2 7± 8 4	7 0± 1 1[b]	—	2801±138	
30± 7 4	7 7± 1 6	—	3010± 98	
191± 6 6	9 5± 1 7	60 7± 9 5	53 2±11 4	
212± 5 9	9 3± 1 4	61 8±11 7	52 6± 8 8	
213± 6 9	9 4± 2 3	63 3± 8 1	62 2±11 4	
194±10 2	9 2± 1 8	61 3± 8 9	55 1±10 2	
198± 6 5	16 5± 4 1	57 4± 9 4	61 5±1° 1[b]	
-04± 5 2	°5 8± 5 7	58 1±11 2	81 1±17 2	
189± 7 2	32 3±10 2	60 7± 8 7	62 7±11 6[b]	
211± 5 9	9 5± 1 3	51 9± 8 7	—	
230± 6 5	11 5± 2 6	57 4± 6 3	—	
219± 4 3	10 3± 2 1	63 9±11 3	—	

Statistical treatment of the results

Standard deviations were calculated for the body and organ weights in the d ferent groups using the conventional statistical formula In determining the significan of the difference between two means Student s t test was applied The probability P w obtained by comparing the calculated value of t with the tabulated values for f variab

In comparing the results of quantitative enzymic determinations the analysis

Table X Hydrolysis rate of *α* naphthyl butyrate naphthol AS acetate with E600 (10 ⁴M, different experimental conditions

Experimental condition		*α* Naphthyl butyrate mμmole/μg N/min		Naphthol AS mμmole/μg
		Mean of test group	Test groups combined	Mean of test group
Male control		3 829	3 829 ± 0 293	1 107
Thyranon	10 days	2 810		0 981
,	20 days	4 087	3 443 ± 0 197	1 242
,	30 days	3 434		1 16[illegible]
MTU	10 days	3 418		*0 910*
,	20 days	6 092	4 85[illegible] ± 0 192	1 [illegible]5[illegible]
,	*30 days*	4 760		1 273
Neo Hombreol	10 days	5 588		1.298
,	20 days	5 998	5 8[illegible]0 ± 0 191[b]	1 442
,	30 days	5 938		1 617
Castration	*10 days*	*5 9[illegible]7*		*1 669*
,	20 days	5 076	5 787 ± 0 187[b]	1 744
	30 days	6 178		1 623
Adreson	10 days	5 367		0 666
,	20 days	4 212	5 105 ± 0 19[illegible]	0 682
,	30 days	5 787		0 816
Adrenalectomy	10 days	4 466		1 431
	20 days	2 868	4 002 ± 0 198	1 046
,	30 days	4 637		*1 563*
Female control		4 778	4 778 ± 0 235	1 046
Progestin	10 days	4 828		1 22[illegible]
,	20 days	4 739	4 8[illegible]0 ± 0 145	1 172
,	*30 days*	*4 9[illegible]3*		*1 078*
Dimenformon	10 days	2 898		0 945
,	[illegible]0 days	2 [illegible]0	2 594 ± 0 146	1 00[illegible]
	30 days	2 401		*0 96[illegible]*
Castration	10 days	6 112		1 153
,	20 days	6 421	6 576 ± 0 143[c]	1 516
,	*30 days*	*7 1[illegible]*		1 [illegible]76

Standard error
$p < 0.05$ ⎫
[b] $p < 0.01$ ⎬ Compared with controls
$p < 0.001$ ⎭

variance was performed the within group variances and the between group variances were thus determined The error risk probability for the variance ratio F was checked from the tabulated distribution for this test variable (Fisher and Yates 1963)

When comparing the significance of the difference between the mean of the individual test group and that of the control group the formula given for the t test was

phthyl acetate and β naphthyl palmitate by the rat adenohypophyseal tissue homogenate in

tate min	α Naphthyl acetate mμmole/μg N/min		β Naphthyl palmitate mμmole/μg N/10 min	
Test groups combined	Mean of test group	Test groups combined	Mean of test group	Test groups combined
1 107 ± 0 015	1 950	1 950 ± 0 123	0 767	0 767 ± 0 109
	1 348		0 658	
1 130 ± 0 010	1 887	1 650 ± 0 082	1 045	0 819 ± 0 072
	1 710		0 706	
	1 599		1 153	
1 16? ± 0 010	3 448	2 555 ± 0 084	1 4?3	1 192 ± 0 074[b]
	2 433		0 991	
	2 842		0 302	
1 457 ± 0 010	2 774	2 8?8 ± 0 08?	0 236	0 294 ± 0 074[b]
	2 868		0 344	
	2 797		0 214	
1 673 ± 0 010	2 709	2 056 ± 0 082	0 841	0 454 ± 0 072
	3 273		0 364	
	2 036		1 443	
0 773 ± 0 010	2 026	2 359 ± 0 084	0 490	0 799 ± 0 074
	2 950		0 624	
	2 350		0 551	
1 365 ± 0 010	1 986	2 32? ± 0 086	0 381	0 598 ± 0 077
	2 666		0 882	
1 046 ± 0 012	2 704	2 704 ± 0 115	0 592	0 592 ± 0 094
	? 607		1 295	
1 157 ± 0 007	2 507	2 547 ± 0 071	1 121	1 151 ± 0 058
	2 5?8		1 036	
	1 616		0 780	
0 970 ± 0 007	1 731	1 668 ± 0 0?2	0 4?2	0 532 ± 0 058
	1 658		0 398	
	3 272		0 5?7	
1 3 4 ± 0 007	3 162	3 290 ± 0 070	0 701	0 569 ± 0 057
	3 432		0 472	

applied The term s is replaced by the square of the within group variance (s_1^2) and the probability P is again obtained from the tabulated values of Student s t test

In this investigation the difference between two means or in the variance ratio is said to be significant if $0\,01 < P \leq 0\,05$ highly significant if $0\,001 < P \leq 0\,01$ and very highly significant if $P \leq 0\,001$

Table X Hydrolysis rate of α naphthyl butyrate naphthol AS acetate with E600 (10^{-4} M) different experimental conditions

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MTU	10 days	3 418		0 910
"	20 days	6 092	4 852 ± 0 19[illegible]	[illegible]
"	30 days	4 760		1 2[illegible]3
Neo Hombreol	10 days	5 588		1 [illegible]98
"	20 days	5 998	5 850 ± 0 191[b]	1 44[illegible]
"	30 days	5 938		1 617
Castration	10 days	5 9[illegible]7		1 669
	20 days	5 076	5 787 ± 0 18[illegible][b]	1 [illegible]44
"	30 days	6 178		1 6[illegible]3
Adreson	10 days	5 367		0 666
"	20 days	4 212	5 10[illegible] ± 0 192[b]	0 68
"	30 days	5 787		0 816
Adrenalectomy	10 days	4 466		1 481
"	20 days	2 868	4 00[illegible] ± 0 198	1 046
"	30 days	4 637		1 563
Female control		4 778	4 778 ± 0 235	1 046
Progestin	10 days	4 828		1 [illegible]
"	20 days	4 739	4 850 ± 0 145	1 172
"	30 days	4 983		1 078
Dimenformon	10 days	2 898		0 945
"	20 days	2 220	2 594 ± 0 146	1 00[illegible]
	30 days	2 401		0 965
Castration	10 days	6 112		1 153
"	20 days	6 421	6 576 ± 0 143	1 516
"	30 days	7 125		1.276

Standard error
$p < 0\ 05$ }
[b] $p < 0\ 01$ } Compared with controls
$p < 0\ 001$ }

variance was performed the within group variances and the between group variances were thus determined The error risk probability for the variance ratio F was checked from the tabulated distribution for this test variable (Fisher and Yates 1963)

When comparing the significance of the difference between the mean of the individual test group and that of the control group the formula given for the t test was

hyl acetate and β naphthyl palmitate by the rat adenohypophyseal tissue homogenate in

e n		α Naphthyl acetate mμmole/μg N/min		β Naphthyl palmitate mμmole/μg N/10 min	
st groups ombined		Mean of test group	Test groups combined	Mean of test group	Test groups combined
97 ± 0 015		1 950	1 950 ± 0 123	0 767	0 767 ± 0 102[a]
		1 348		0 658	
30 ± 0 010		1 887	1 650 ± 0 082	1 045	0.819 ± 0 072
		1 710		0 706	
		1 599		1 153	
6[illegible] ± 0 010		3 448	2 555 ± 0 084	1 4[illegible]3	1 192 ± 0 074[a]
		2 433		0 991	
		2 842		0 302	
[illegible] ± 0 010		2 774	2 823 ± 0 082	0 236	0 [illegible]94 ± 0 074[a]
		2 868		0 344	
		2 797		0 214	
73 ± 0 010		[illegible] 709	2 956 ± 0 082	0 841	0 454 ± 0 [illegible]
		3 273		0 364	
		2 036		1 443	
73 ± 0 010		2 026	2 359 ± 0 084[b]	0 490	0 7[illegible] ± [illegible]
		2 950		0 624	
		2 350		0 551	
65 ± 0 010		1.986	2 322 ± 0 086	0 381	[illegible]
		2 666		0 882	
46 ± 0 012		2 704	[illegible] 704 ± 0 115	0 59[illegible]	[illegible]
		2 607		1 29[illegible]	
57 ± 0 007		2 507	[illegible] 547 ± 0 071	1 121	[illegible]
		2 528		1 036	
		1 616		0 780	
970 ± 0 007		1 731	1 668 ± 0 072	0 4[illegible]2	[illegible]
		1 658		0 398	
		3 [illegible]72		0 527	
324 ± 0 007		3 162	3 [illegible]90 ± 0 070	0 701	[illegible]
		3 432		0 4[illegible]2	

applied The term s is replaced by the square of the within [illegible] and the probability P is again obtained from the tabulated values of [illegible]

In this investigation the difference between two means [illegible] said to be significant if $0 01 < P \leq 0 05$, highly significant if [illegible] and very highly significant if $P \leq 0 001$

Results and discussion

Table IX gives the initial and final body weight of the animals in each individual group as well as the mean weights of the anterior pituitary glands adrenals testes or ovaries and the thyroid glands the latter only in the control male group and after thyroxin and MTU treatment The significance of the changes is given for the organ weights The changes are in agreement with those previously reported in the literature under identical experimental conditions

The mean hydrolysis rates of the four different substrates in the various test groups are given in Table X The results show that in most of the experimental groups no apparent trend toward increasing or decreasing direction was found during the experimental intervals of 10 20, and 30 days For this reason the results of the three subgroups with identical treatment were combined and in each case treated as an entity, although the duration of the treatment was different

When the esterolytic activity between the male and female control groups were compared it was found that the hydrolysis was significantly stronger in the female control group when α NA or α NB were used as substrates No significant differences were, however, found in the hydrolysis of N AS and β NP

The analysis of variance was separately performed for the male and female test groups in the experiments using the four different substrates The results of the analysis are given in Tables XI—XIV and they show that there are very highly significant differences between the groups in the hydrolytic activities

α Naphthyl butyrate — The hydrolysis of β NB was highly significantly increased after castration both in the male and female rats This increase presumably represents partly the fine granular activity in both gonadotropic cells in fresh frozen sections and partly the pericytic activity in fixed sections Estrogen treatment had a contrary effect on both these activities and this is shown as a very highly significant decrease in the quantitative determination *Progesterone was without effect on the total* hydrolytic activity but testosterone had an increasing effect which presumably reflects the increased activity in FSH cells and STH cells The simultaneous decrease of the esterase activity in LH cells has however an equalizing effect on the total hydrolytic activity

Thyroxin treatment did not cause any significant change in the hydrolytic activity although in the TSH cells in fresh frozen sections a decrease of activity was evident The increase after methylthiouracil treat

Table XI α Naphthyl butyrate (mμmole α naphthol/μg N/min)

Male groups Analysis of variance Variation	Q	f	s	F	P
Between groups	155 79	6	25 965	13 49	<0 001
Within groups	363 76	189	1 925		
Total	519 55	195			
Female groups Analysis of variance Variation	**Q**	**f**	**s²**	**F**	**P**
Between groups	286 40	3	95 470	78 84	<0 001
Within groups	152 56	126	1 211		
Total	438 96	1 9			

ment was significant and presumably due to the increased esterase activity in TSH cells in fresh frozen sections The increase in the hydrolysis was also significant after cortisone treatment but the histochemical counterpart for this increase is difficult to point out This increase may however, be due to the closer packing of the active gonadotropic cells after the shrinkage of the ACTH cells No significant change was found after adrenalectomy

Naphthol-AS acetate — In the total pituitary homogenate the E s ns E was found to represent only about 6 % of the hydrolytic activity against N AS (Table VII) E r ns L activity hydrolyzing N AS was markedly increased after castration both in males and in females The decrease after estrogen treatment was also very highly significant Progesterone increased significantly this activity These changes are in agreement with those observed histochemically in the gonadotropic cells

No significant effect on the total hydrolysis of N AS was obtained after thyroxine or MTU treatment This is not in correlation with the histochemical changes in the E r ns E activity in the TSH cells but it is acceptable because of the simultaneous opposite change in the esterase activity in the STH cells The increase after testosterone treatment is most probably in connection with the increased hydrolytic activity in STH cells

Adrenalectomy caused a very highly significant increase while cortisone treatment markedly decreased N AS hydrolysis Histochemically identical changes were observed in the coarse granulated cytoplasmic activity in the ACTH cells

Table XII Naphthol AS acetate + E600 (10^{-5} M) (mμmole naphthol AS/μg N/min)

Male groups					
Analysis of variance					
Variation	Q	f	s	F	P
Between groups	14 64	6	2 440	48.22	<0 001
Within groups	9 56	189	0 051		
Total	24 20	195			
Female group					
Analysis of variance					
Variation	Q	f	s^2	F	P
Between groups	5 48	3	1 827	56 39	<0 001
Within groups	4 08	126	0 032		
Total	9 56	129			

α Naphthyl acetate — This substrate is hydrolyzed by both E s ns Es and E i ns Es and changes in both categories are reflected in the hydrolysis An increase was also obtained after castration both in males and females just as with the previously reported substrates A very highly significant decrease was caused by estrogen treatment as was anticipated on the basis of the histochemical results A marked increase was also found after methylthiouracil and testosterone treatment Progesterone was without any significant effect while an increase was shown both after cortisone treatment and after adrenalectomy These changes are relevant to the findings obtained with α NB and N AS when the hydrolytic activities are combined

Table XIII α Naphthyl acetate (mμmole α naphthol/μg N/min)

Male group					
Analysis of variance					
Variation	Q	f	s	F	P
Between groups	37 97	6	6 328	17 31	<0 001
Within groups	69 09	189	0 366		
Total	10 06	195			
Female groups					
Analysis of variance					
Variation	Q	f	s	F	P
Between groups	48 02	3	16 006	54 61	<0 001
Within groups	36 93	126	0 293		
Total	84 95	129			

Table XIV β Naphthyl palmitate (mμmole β naphthol/μg N/10 min)

Variation	Q	f	s^2	F	P
Male group					
Analysis of variance					
Between groups	14 42	6	2 403	8 33	<0 001
Within groups	53 36	189	0 288		
Total	67 78	195			
Female groups					
Analysis of variance					
Variation	Q	f	s^2	F	P
Between groups	9 14	3	3 047	15 61	<0 001
Within groups	24 59	126	0 195		
Total	33 73	129			

Adrenalectomy caused only a slight increase in the hydrolysis of these substrates which was not significant but when both activities are partaking in the hydrolysis of α NA, a significant change may be expected After cortisone treatment the hydrolysis of α NB was increased while that of N AS was decreased Numerically the increase of the F s ns Es was greater and contributed also more markedly to the hydrolysis of α NA

β Naphthyl palmitate — The most significant change in the hydrolysis of β NP was observed after progesterone treatment which caused a marked increase Methylthiouracil had also an increasing effect while a decrease was apparent after testosterone treatment and after castration in males The hydrolysis in other groups was not significantly altered

The site of the hydrolytic activities toward β NP separated by starch gel electrophoresis and DEAE cellulose chromatography could not be visualized in the histochemical study The fractionation studies however, showed that the hydrolytic activities which split the short chained carboxylic esters contribute only weakly to the hydrolysis of β NP Thus a different category of enzymes is mainly described in the quantitative studies The results obtained in these studies also show that the changes obtained are not concomitant with β NP and those obtained with other substrates

Table XII Naphthol AS acetate + E600 (10^{-4} M) (mμmole naphthol AS/μg N/min)

Male groups Analysis of variance Variation	Q	f	s	F	P
Between groups	14 64	6	2 440	48 22	<0 001
Within groups	9 56	189	0 051		
Total	24 20	195			

Female group Analysis of variance Variation	Q	f	s	F	P
Between groups	5 48	3	1 827	56 39	<0 001
Within groups	4 08	126	0 032		
Total	9 56	129			

α Naphthyl acetate — This substrate is hydrolyzed by both E s ns Es and E r ns Es and changes in both categories are reflected in the hydrolysis An increase was also obtained after castration both in males and females just as with the previously reported substrates A very highly significant decrease was caused by estrogen treatment as was anticipated on the basis of the histochemical results A marked increase was also found after methylthiouracil and testosterone treatment Progesterone was without any significant effect while an increase was shown both after cortisone treat ment and after adrenalectomy These changes are relevant to the findings obtained with α NB and N AS when the hydrolytic activities are combined

Table XIII α Naphthyl acetate (mμmole α naphthol/μg N/min)

Male groups Analysis of variance Variation	Q	f	s	F	P
Between groups	37 97	6	6 328	17 31	<0 001
Within groups	69 09	189	0 366		
Total	107 06	195			

Female groups Analysis of variance Variation	Q	f	s	F	P
Between groups	48 02	3	16 006	54 61	<0 001
Within groups	36 93	126	0 293		
Total	84 95	129			

Characteristic features for different cells have been searched also in inactive or incompletely differentiated stages with the histochemical enzyme reaction Thus endocrine cells producing steroid hormones have been identified with a histochemical enzyme reaction although the hormones of these cells have not yet been demonstrated or the cells are still morphologically undifferentiated (Niemi and Ikonen 1962) The biosynthesis of the adenohypophyseal hormones includes, unfortunately no such specific enzyme catalysis which could make the histoenzymological differentiation of the cells possible In the oxidative metabolism of various cell types only minor differences have been found which show that the basophilic cells are usually most active but the other cells are not totally devoid of these activities (Bleicher et al 1961 Balogh and Cohen 1962 Sobel 1964 Fand 1965)

There is a large number of esterases in different endocrine glands, their activity is quite intimately connected to hormone production (Allen 1957 Ikonen 1965 Konig 1965 Niemi et al 1966) Reports have also been published earlier on the esterase activity of the adenohypophyseal cells although quite different statements on their localization have been presented (Dumont 1956a b Fand 1955 1961 Pearse 1956 Lojda 1960 Lojda and Schreiber *1960, 1964*, Sobel *1962* Jirasek *1963* Wächtler and Pearse 1966) This at least partly can be explained due to species differences

The present studies show that in the rat pituitary gland there is a positive correlation of the L s ns E activity to the production of FSH LH TSH and STH The subcellular site of this reaction was not possible to specify with certainty It was located evenly over the cytoplasm and solubilized by Triton X 100 The latter finding is the same as made by Carruthers and Baumler (1962) on the E s ns Es in liver microsomes The microsomal particles are known to be the site of intracellular protein synthesis The role of this type of esterase in the glycoprotein and protein hormone synthesis cannot be specified due to the incomplete knowledge of the mechanism of hormone production It may be assumed however that this type of carboxylic esterase does not directly act in the building up of a protein molecule although the hydrolytic enzymes are claimed to act as transferases (cf Hofstee 1960) The site of the esterase activity in the microsomal membranes (Chaveau et al 1962) presupposes that these enzymes are somehow connected with the treatment of the raw material for hormone synthesis

Another E s ns E activity in fresh frozen sections was mainly located in the STH cells where it was mostly confined to the cellular periphery This type of esterase activity seems to be somewhat more intensely attached

to the intracellular particles, but can be dissolved also by Triton X 100 at higher concentrations Part of this activity may reside in the microsomal particles but other cellular components including the cell membrane may be equally in question STH cells in the active phase contain densely arranged lamellar endoplasmic reticulum in the periphery of the cell body (Herlant 1963 Yoshimura and Harumiya 1965) The secretory granules of these cells are transferred to the cell membrane and adhere to it the limiting membrane is dissolved possibly by an intracellular enzyme activity (Farquhar 1961, Weiss 1965 Yoshimura and Harumiya 1965), which may be an esterolytic enzyme After the release of the secretory granule out of the cell the granule membrane is left inside the cell and a ring of ribosomes is attached to it (Weiss 1965) These particles may thus also be responsible for the peripheral activity In the gonadotropic cells the secretory granules loose their contents intracellularly and the dispersed material is claimed to be eliminated physicochemically (Yoshimura and Harumiya 1965)

Pearse (1956) reported that in fresh frozen sections of the human pituitary gland an intense esterase activity was found in the pericytic cells when indoxyl acetate was used as substrate The present study shows that in the rat pituitary gland no such activity was observed but in formalin fixed sections the same cell type revealed a strong E s ns E with α NB as substrate but not with 5 BIA The pericytic activity in the human pituitary was found to be quite variable (Pearse 1956) and this was also true in the rat pituitary gland A positive correlation between this activity and the increase in the gonadotropic hormone secretion was however, clearly shown The phagocytizing functions of these cells give reason to suppose that this soluble esterase activity is connected with the digestion processes of the engulfed lipid material There is good reason to object to the idea of Pearse (1956) that this pericytic activity is derived from phagocytosis of the esterase bearing protein Another esterase type was found however in the same cells after delivery for a short time period This F i ns E had the same characteristics as the intracellular coarse granulated activity in gonadotropic cells and it is supposed that this activity may be derived from the parenchymal cells after it has become useless for the cellular functions

The role of the esterases in the secretory process of the pituitary gland could be better evaluated if the mechanism of the secretion would be worked out Cardell (1962) working on *Triturus*, described buds of the parenchymal cells protruding during hormonal release, into the cytoplasm of either a pericapillary cell or a stellate pericytic cell The bud is pinched

of to become a vacuole within the invaded cell The cell then breaks down the granules and releases the hormone into its own cytoplasm from which it enters the pericapillary space The same kind of budding was also found in the pituitary gland of the platyfish, but the secretory granule itself was released into the perisinusoidal space and may move through the capillary endothelium or fenestrae in it into the capillary lumen in invisible form (Weiss 1965) The remnants of the parenchymal cell buds may, however, be acted upon by the pericytic cells and in the digestion of these cell components the pericytic esterase activity may equally well be involved

A strong granular esterase activity in the mucoid cells of the paraffin embedded human hypophysis was found to reside in the Golgi zone (Pearse 1956) but its sensitivity to E 600 was not accurately specified In the present study an I r ns E was confined to the same zone and a marked increase in this activity was connected with the increased secretory function This effect was most intense in both gonadotropic cells after castration which is known to be connected with the increased content of FSH and LH in the pituitary gland (Greep and Chester Jones 1950 Cozens and Nelson 1961) An increase was also shown in FSH cells but not in LH cells after testosterone treatment which is in agreement with the dissociated action of this hormone on the secretory processes of FSH and LH (Hoogstra and Paesi 1957, Paesi et al 1953, 1959) A further confirmation for the contribution of this esterase to secretory function was obtained in young rats which had a large number of peripheral cells with an active microsomal F s ns E but were almost devoid of the I r ns E These cells are probably already active in the synthesis of FSH but at this stage they do not release their hormones

High F r ns E activity was shown in the Golgi area of the thyrotropic cells too This finding is in agreement with that of Sobel (1962), who concluded that the actual site of this activity may be in the lysosomes Both light and electron microscopic studies have confirmed the increase of the lysosomal particles in the Golgi area during augmented secretory activity of gonadotropic and thyrotropic cells (Yoshimura et al 1964, Yoshimura and Harumiya 1965)

A number of electron microscopic studies have shown that the Golgi apparatus is intimately connected with *the formation* of the secretory granules (Ichikawa 1959 Farquhar 1961, Gabe and Arvy 1961 Maillard 1963 Weiss 1965) The Golgi zone is not involved in the actual synthesis of the hormone but acts as a 'condensation center', where the granule membranes are possibly formed of the Golgi membranes Novikoff et al

(1962) have suggested that the lysosomal hydrolases are involved in the condensation or other aspects of the secretory material accumulation

The fractionation studies showed that the total number of enzymic activities in the rat pituitary gland was greater than could be expected from the histochemical studies Some of the enzyme double bands separated in the starch gel electrophoresis were quite similar in their inhibitor characteristics and substrate specificity Those may be called 'isozymes according to Markert and Møller (1959) or technical artefacts according to Cann (1966), until our growing knowledge of the multiple enzyme systems shows that these activities *in vivo* have really different cellular locations and differences also in characteristics other than their migration in electrophoresis

Almost all the different esterase activities visualized in the starch gel slab could be correlated with separate activities in the histological sections There were still other esterases more specific for the long chained naphthol esters that could not be demonstrated in sections, but were separated both in the starch gel electrophoresis and DEAE cellulose chromatography The ignorance of the cellular site of these enzymes leaves their possible role unclear, although significant changes were found in the total activity using β NP as substrate The quantitative studies with other substrates were properly in agreement with the changes found in histochemical studies during adequate experimental conditions

SUMMARY

The histology and the esterase activity of the rat anterior pituitary gland were studied in material from about 2000 animals The production of pituitary hormones was altered in the following experimental conditions castration adrenalectomy feeding with thyroxine and methylthiouracil, injection with testosterone cortisone, progesterone estrogen and reserpine, female rats during pregnancy and lactation and immature rats To elucidate the histologically characteristic features of the six different parenchymal cell types as well as other cellular constituents, the following staining techniques were used periodic acid Schiff orange G aldehyde fuchsin alcian blue at pH 3 0 and 0 2 tetrachrome, acid fuchsin aniline blue aldehyde thionin, and azocarmine orange G

Carboxylic esterases were studied histochemically in fresh frozen and formalin fixed sections using acetyl and butyrylthiocholine as substrate for the demonstration of cholinesterases and α naphthyl acetate, α naphthyl propionate α naphthyl butyrate α naphthyl valerate, α naphthyl caprylate β naphthyl laurate, β naphthyl myristate β naphthyl palmitate naphthol AS acetate naphthol AS D chloroacetate, 5 bromoindoxyl acetate and thio acetic acid for the demonstration of non specific esterases The esterases were classified with the aid of different substrates and modifier substances into four types acetylcholinesterase (AChE) non specific cholinesterase (ns ChE), E600 sensitive non specific esterase (E s ns E), and E600-resistent non specific esterase (E r ns E)

Carboxylic esterases of the total anterior pituitary homogenate were further fractionated by the following methods separation into soluble and particle bound components, starch gel electrophoresis and DEAE cellulose column chromatography The total esterase content of the homogenates was finally determined with a number of substrates and changes during some experimental conditions were elucidated

Histological and histochemical cell characteristics

Somatotropic cells were orangeophilic in the azocarmine orange G method degranulated after methylthiouracil feeding and the most abun

dant cell type in the young rats The cellular periphery in fresh sections had an I s ns I , which increased during augmented hormone production and decreased after methylthiouracil treatment In fixed sections a coarse granular perinuclear I r ns F was shown, which clearly increased in young rats and after testosterone treatment

Lactotropic cells were carminophilic in the azocarmine-orange G method, erythrosinophilic in the tetrachrome method most abundant during lactation and after estrogen and reserpine treatment and lacking in young rats. They showed negligible or no L s ns F and E r ns E even during augmented hormonal production

Corticotropic cells contain erythrosinophilic granules in the tetrachrome method, they are present in large numbers after adrenalectomy, but almost totally absent after cortisone treatment There was a weak peripheral E s ns F without any apparent change in experimental conditions, and a moderate coarse granular perinuclear activity in fixed sections, which increased after adrenalectomy

Thyrotropic cells are polygonal, stain red with aldehyde fuchsin, blue with aldehyde-thionin stain green with alcian blue at pH 0 2, and are located in the central part of the gland Evenly distributed fine-granular cytoplasmic I s ns F increased in these cells after methylthiouracil feeding and decreased after thyroxin treatment Coarse-granular perinuclear E r ns F increased also in fixed sections after methylthiouracil and decreased after thyroxin These cells contained in addition a weak diffuse ns ChE with no apparent changes in experimental conditions

FSH cells were round or oval with a perisinusoidal distribution in the peripheral parts of the gland including the 'sex zone', and lining the ventrocaudal edge of the gland in young rats They were PAS-positive, aldehyde fuchsin negative, and violet stained in the alcian blue pH 0 2 method Strong I s ns I was present in the whole cytoplasm even in young animals increased after castration and testosterone treatment, was unaffected by progesterone and markedly decreased after estrogen Strong coarse granular perinuclear I r ns I in fixed sections increased after castration was unaffected by testosterone and progesteron, but was almost absent in the young rats

IH or ICSH cells were round or oval perisinusoidal cells in the central part of the gland They were PAS positive, aldehyde fuchsin negative, and red in the alcian blue pH 0 2 staining Their number was markedly increased after castration and during pregnancy, and decreased after testosterone and progesterone they were almost absent after estrogen treatment Cytoplasmic fine granular E s ns F in these cells was greatly in

creased after castration and during pregnancy, and decreased after testosterone progesterone and estrogen Perinuclear E r ns E in fixed sections was increased after castration and during pregnancy and absent after estrogen treatment

Pericytic cells were fusiform or stellate, blue stained with the acid fuchsin aniline blue method and intimately related to the gonadotropic cells after castration No esterase activity was present in fresh frozen sections but in fixed sections a strong E s ns E was found with α naphthyl butyrate as the preferred substrate The activity is markedly increased during augmented secretory activity of the gonadotropic cells and absent after estrogen treatment During the first days after delivery these cells in the female pituitary gland also have E r ns E probably derived from the parenchymal cells

Capillary endothelial cells showed a weak granular activity with thiocholine esters it was not affected by the modifiers used and is consequently different from the esterases classified earlier Weak E r ns E was shown only with acetic acid esters in fixed sections No apparent changes were observed in either of these activities in the experimental conditions

Cleft epithelial cells between the anterior and intermediate lobes showed a weak ns ChE with no apparent changes under the experimental conditions The strong E s ns E in these cells in fixed sections was markedly increased after castration and adrenalectomy when also the holocrine secretion of these cells was increased

Fractionation of the esterases

Soluble and particle bound enzymes Both E s ns Es and E r ns Es were present in the soluble and particulate fractions after centrifugation of the pituitary homogenate in 0 25 M sucrose solution A great portion of the particle bound activity was readily solubilized by Triton X 100, although some activity still remained in the particulate fraction Activity which hydrolyzed β naphthyl palmitate was mainly particulate and only partially solubilized by Triton X 100

Starch gel electrophoresis Eleven activity bands were shown when the starch slabs were stained by the histochemical esterase technique Four of them were E r ns Es the others were E s ns Es Most of the electrophoretic fractions could be identified with the activities demonstrated in the tissue sections Two separate activities splitting β naphthyl palmitate and two main hydrolytic activities for thiocholine esters were found using

the eluates of the transversely cut starch gel blocks One was not separable from the second β naphthyl palmitate activity, both were resistent to eserine and E600

DEAE cellulose column chromatography Separation of the F 1 ns I peak from three partially separated E s ns E peaks was achieved These activities were also separable from the ns ChE peak one peak preferentially hydrolyzing long chained naphthol esters as well as from another peak splitting both thiocholine esters and long chained naphthol esters Correlation was made with the previously separated proteolytic activities when the same method was used

Quantitative changes in total esterase activity

The changes in the hydrolysis of α naphthyl acetate α naphthyl butyrate naphthol AS acetate with L600 and β naphthyl palmitate in the experimental conditions (male rats castrated or adrenalectomized, injected with testosterone or cortisone fed with methylthiouracil or thyroxine and female rats castrated or injected with progesterone or estrogen) were compared with male and female controls Significant changes were obtained with all these substrates and they were generally in agreement with those obtained in the histochemical studies Observations with β naphthyl palmitate could not be related to any histochemical results The changes shown with this substrate were not analogous to those obtained with other substrates which is most probably due to the different enzyme categories in the hydrolysis of different substrates

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STUDIES ON VASCULAR PERMEABILITY IN PERIPHERAL NERVES

2 Distribution of circulating fluorescent serum albumin in rat sciatic nerve after local injection of 5 hydroxytryptamine, histamine and Compound 48/80

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The vascular architecture of peripheral nerves has been described by several authors (Adams 1942 Sunderland 1945 Richards 1951, Waksman 1961 Edshage 1964) In the epineurium and the perineurium there are vascular networks which communicate with a plexus of small anastomosing blood vessels in the endoneurium of the nerves

There seems to be a difference in permeability between the epineurial and the endoneurial blood vessels of rat sciatic nerves under physiological conditions (Olsson 1966 a) After intravenous injection of fluorescent labelled serum albumin fluorescence appeared both in the lumen and in the walls of epineurial blood vessels In addition a diffuse faint extra vascular fluorescence was observed which extended to the innermost parts of the epineurium and the perineurium In the endoneurium however, fluorescence was only observed in the lumen of blood vessels No fluorescence was detected in or outside the walls of these blood vessels

Traumatic lesions of rat sciatic nerves are accompanied by a rapid and marked increase in the permeability of both endoneurial and epineurial blood vessels for fluorescent labelled serum albumin (Olsson 1966 a) The most marked exsudation is observed at the site of the lesion during the initial hours after the trauma Simultaneously, the exsudated fluorescent albumin spreads chiefly into the distal endoneurium Corresponding observations have recently been made by Mellick (1966) who found that physical trauma to chicken sciatic nerves is associated with a rapid increase in the wet weight most pronounced in the segments adjacent to and distal of the lesion Quantitative determinations of intravenously injected J^{131} labelled serum albumin revealed a marked increase of labelled albumin in the corresponding segments

Increased vascular permeability for serum proteins and formation of protein rich exsudate are cardinal features of the acute inflammatory process (Spector & Willoughby 1963 1964 1965 Majno 1964, Wilhelm 1962 1965) It is generally held that this increased vascular permeability can be induced by direct damage to vascular walls and by liberated endogenous chemical mediators

Of various mediators histamine and 5 hydroxytryptamine (5 HT serotonin) have the capacity to induce a rapid transient increase in the permeability of blood vessels in several tissues (Spector & Willoughby

1963 1964, 1965, Majno 1964 Wilhelm 1962 1965) The central nervous system appears however, to be an exception Thus Broman and Lindberg-Broman (1945) reported that cerebral blood vessels entirely resisted the action of histamine, even in large doses The effect of histamine and 5 hydroxytryptamine on blood vessels in peripheral nerves does not seem to have been described previously

Unlike the nerve fibres of the brain those of the peripheral nerves are separated by connective tissue having a substantial extracellular space (Thomas 1963, Gamble 1964, Waggener Bunn & Beggs 1965) Mast cells are normally present in the connective tissue of the nerves both in the epineurium the perineurium and the endoneurium (Enerback Olsson & Sourander 1965) Those cells most probably store the major part of the histamine and 5 hydroxytryptamine normally contained in rat peripheral nerves (Kwiatkowski 1943 Torp 1961, Olsson 1965 Anden & Olsson 1966)

Numerous authors have observed that the onset of the acute inflammatory process in several tissues of the rat is associated with degranulation of mast cells (Maximow 1904 Ernst 1926 McGovern 1957, Gustavsson & Cronberg 1957 Spector & Willoughby 1959 Sheldon & Bauer 1960) These cells are highly responsive and fragile A large number of stimuli e g mechanical trauma heat cold ionizing radiation and several chemical and biological agents have the capacity to induce degranulation of mast cells and liberation of histamine and 5 hydroxytryptamine (Moran, Uvnas & Westerholm 1962 Uvnas 1964) One of the most potent mast cell degranulating agents is Compound 48/80 a condensation product between paramethoxyphenethylmethylamin and formaldehyde (Baltzy Buck, deBeer & Webb 1949) Several investigators have also observed that local injection of this compound into various tissues of the rat is associated with degranulation of mast cells and increased vascular permeability leading to the formation of oedema (Rowley & Benditt 1956 Sparrow & Wilhelm 1957 Gotzy & Kato 1964 Rowley 1964)

The aim of the present study was to investigate the effect of the mast cell amines histamine and 5 hydroxytryptamine on the vascular permeability in peripheral nerves In addition the effect of the histamine liberator Compound 48/80 was studied in the same way

MATERIALS AND METHODS

Vascular permeability changes was demonstrated by fluorescence micro scopic tracing of intravenously injected serum albumin tagged with Evans blue according to Steinwall and Klatzo (1965) Histamine 5 hydroxytryptamine and Compound 48/80 were injected into the endoneurium or applied to the surface of the epineurium of rat sciatic nerves Some of the animals were pretreated with a histamine antagonist mepyramine maleate others with a serotonin antagonist methysergide bimaleinate

Previous studies have revealed that mepyramine maleate has a strong antihistaminic action (Spector & Willoughby 1965 Swanes 1965) It has also been shown that methysergide bimaleinate has a marked antagonistic effect on the serotonin induced oedema in the skin of rats (Fanchamps Doepfner Weidmann & Cerletti 1960) and mice (Swanes 1965)

Animals

164 Sprague Dawley rats of both sexes were used weighing between 250 and 350 g They were kept on a diet of commercial rat pellets and water ad libitum The injections were performed under ether anesthesia At suitable times, varying between five min and six hrs after the injections rats were killed by bleeding from a cut in the heart

I Microinjections into the endoneurium of sciatic nerves

Injections into the endoneurium of sciatic nerves were performed as previously described (Olsson 1966 b) The nerves were exposed high up in the thigh A very fine glass capillary connected via a short plastic tube to a micrometer syringe[1]) was used for the injections The glass capillary was inserted distally into the nerves Both sciatic nerves were used in each rat with less than two min delay between the injections

Groups of three to five rats received Evans blue albumin (EBA)[2]) either before or after injection of the permeability changing agents as stated below

Controls

Groups of rats were injected with EBA[2]) immediately followed by a single endoneurial injection of 0 002, 0 004 or 0 006 ml Ringer solution

[1]) Agla Micrometer Syringe Burroughs Wellcome & Co England

[2]) Evans blue albumin abbreviated EBA (Klatzo & Steinwall 1965)

respectively They were killed after 15 min Groups of rats injected with 0 002 ml Ringer solution were also killed 2 and 6 hrs after the injection

In subsequent experiments the permeability changing agents were dissolved in Ringer solution For microinjections into the endoneurium a volume of 0 002 ml was used in all the experiments

5-hydroxytryptamine

Groups of rats were injected with EBA immediately prior to the local injection of 1 μg 5 hydroxytryptamine[3]) They were killed after 15 min and 2 hrs respectively

The duration of the permeability increasing capacity of 5 hydroxytryptamine was studied in one group of rats by the injection of 1 μg 5 hydroxytryptamine into the nerves followed 15 min later by EBA The animals were killed 30 min after the last injection

Two groups received subcutaneously 0 1 mg methysergide[4]) per 100 g body weight 30 min later EBA immediately followed by the injection of 1 μg 5 hydroxytryptamine They were killed 30 min after the last injection

Histamine

Groups of rats were injected with EBA immediately prior to the local injection of 1 μg histamine[5]) They were killed after 15 min and 2 hrs respectively

Other groups of rats were injected with EBA immediately prior to the local injection of 10 μg histamine They were killed after 15 min and 2 hrs respectively

The duration of the permeability increasing effect of histamine was studied in one group of rats injected with 10 μg histamine into the nerves followed 15 min later with EBA The rats were killed 30 min after the last injection

Two groups received subcutaneously 0 1 mg mepyramine[6]) per 100 g body weight, 30 min later EBA immediately followed by the injection of 10 μg histamine The rats were killed 30 min after the last injection

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[3]) 5 hydroxytryptamine serotonin creatinin sulphate obtained from Sigma Chemical Co St Louis U.S A

[4]) Methysergide bimaleinate 1 methyl d lysergic acid butanolamide Deseril® kindly supplied by Sandos Ltd Basel Switzerland

[5]) Histamine dihydrochloride obtained from Fluka AG Chemische Fabrik Switzerland

[6]) Mepyramine maleate Anthisan® kindly supplied by May & Baker Ltd Dagenham England

Compound 48/80

Groups of rats were injected with EBA immediately followed by the local injection of 10 μg Compound 48/80[7]) They were killed 15 min and 2 hrs after the last injection

The duration of the permeability increasing capacity of Compound 48/80 was studied in groups of rats locally injected with 10 μg Compound 48/80 followed 15 min and 2 hrs later respectively by the injection of EBA They were killed 30 min later

Groups of rats received both methysergide and mepyramine subcutaneously (0 1 mg per 100 g body weight) 30 min later EBA immediately followed by the local injection of 10 μg Compound 48/80 They were killed 15 min and 2 hrs later respectively

II Local application to the epineurium of exposed sciatic nerves

The sciatic nerves were exposed by making small incisions high up in the thigh Ringer solution was first applied around the right sciatic nerve immediately followed by injection of the permeability changing agents dissolved in Ringer solution around the left nerve The dosages were the same as those used by Rowley and Benditt (1956) and Rowley (1964) in studies on rat paws i e 0 1 ml 5 hydroxytryptamine (2 μg/ml) histamine (400 μg/ml) and Compound 48/80 (10 μg/ml)

The experimental groups corresponded to those used in the experiments with microinjections into the nerves Groups of rats were injected with EBA immediately followed by the local injections around the nerves They were killed after 5 min and 2 hrs respectively The duration of the permeability changing capacity was studied on other groups of rats which received local injections first, followed 15 min later by the injection of EBA These rats were killed 30 min later Other groups again were pre treated with methysergide and mepyramine singly or in combination 30 min before the injections of EBA and the permeability changing agents They were killed either 15 min or 2 hrs later

Demonstration of vascular permeability

The technique for demonstrating vascular permeability has been described in detail elsewhere (Olsson 1966 a) Briefly each rat received intravenously 1 ml per 100 g body weight of a solution containing Evans

[7]) Compound 48/80 a condensation product between paramethoxyphenethylmethylamine and formaldehyde kindly supplied by the Wellcome Research Laboratories Beckenham Kent England

blue[8]) in 5 % bovine albumin [9]) The sciatic nerves were fixed in 5 % formalin for 24 hrs Frozen longitudinal sections 10 μ thick, from various levels of the nerves were mounted in 50 % aqueous glycerin and immediately studied in a fluorescence microscope (equipped with a dark field condensor and light from an OSRAM HBO 200 W high pressure mercury lamp) The light was filtered through a Schott BG 12/3 mm filter In the tubes the emitted light was filtered through a combination of a Schott OG 4 and a GG 4 filter

The technique used here is based on the recent finding that EBA emits a strong red fluorescent light which permits detailed tracing of this substance in thin frozen sections (Steinwall & Klatzo 1965 Klatzo & Steinwall 1965)

[8]) Evans blau E Merck A G Darmstadt Germany

[9]) Nutritional Biochemicals Corporation Cleveland Ohio U.S.A

RESULTS

I Microinjections into the endoneurium of sciatic nerves

Controls

The injection of 0 002 ml Ringer solution did not appreciably disturb the distribution of fluorescent albumin (EBA) in the endoneurium compared with the distribution in normal nerves as described in a previous paper (Olsson 1966 a) All rats displayed only slight local changes at the site of the injection with small droplets of red fluorescent products appearing in the walls of a few endoneurial blood vessels (Fig 1) The rest of the endoneurium appeared normal Injections of 0 004 and 0 006 ml Ringer solution produced more pronounced exsudation at the site of the injection with a diffuse faint extravascular fluorescence around the endoneurial blood vessels

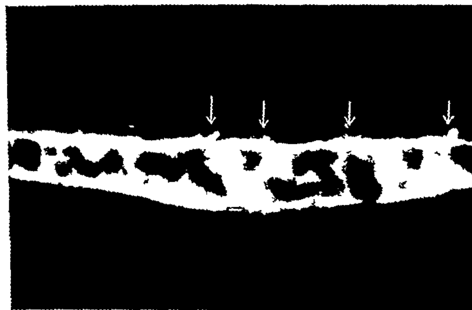


Fig 1 Endoneurial injection of Ringer solution (control) into rat sciatic nerve preceded by intravenous injection of Evans blue albumin (EBA) Fluorescence in the lumen of an endoneurial blood vessel and a few droplets in the vascular walls (arrows)

5-hydroxytryptamine

Local injection of 5 hydroxytryptamine preceded by intravenous injection of EBA resulted in a marked exsudation of fluorescent material into the endoneurium of all rats At the site of the injection an indistinct limited zone with a bright red diffuse fluorescence was observed between the nerve fibres (Fig 2) The amount of extravascular fluorescent material was most pronounced at the site of the injection In parts remote from the injection site, a meshwork of fluorescent material appeared in the vascular walls and in addition there were multiple small fluorescent droplets just outside the endoneurial blood vessels (Fig 3) The extension of the exsudated material did not differ between rats studied 15 min and 2 hrs after the injection

When EBA was injected 15 min after the local injection of 5 hydroxytryptamine there was only a faint fluorescence in and just outside vascular walls at the site of the injection while the other parts of the nerves appeared normal (Fig 4)

Rats treated with methysergide before the local injection of 5-hydroxytryptamine displayed fluorescence in the lumen of endoneurial blood vessels and small fluorescent droplets in the walls of endoneurial blood vessels at the site of the injection (Fig 5)



Fig 2 Microinjection of 5 hydroxytryptamine preceded by intravenous injection of EBA Vast intense fluorescense between the nerve fibres in the indoneurium

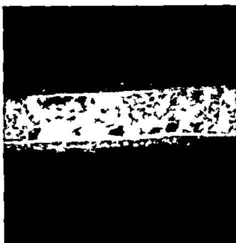


Fig 3 Leaking blood vessel in the endoneurium remote from the site of injection of 5 hydroxytryptamine



Fig 4 Intravenous injection of EBA 15 min after local injection of 5 hydroxytryptamine Endoneurial blood vessel with normal appearance



Fig 5 Pretreatment with methysergide before injections of EBA and 5 hydroxytryptamine Endoneurial blood vessel with normal appearance

Thus compared with local injections of Ringer solution the injections of 5 hydroxytryptamine induced a markedly increased permeability of endoneurial blood vessels The increased permeability was of early onset and brief duration and could be inhibited by pretreatment of the rats with the 5 hydroxytryptamine-antagonist methysergide bimaleinate

Histamine

Injections of 1 µg histamine resulted in a discreate leakage of fluorescent material around endoneurial blood vessels at the site of the injection in twelve nerves The remaining four nerves displayed the same distribution of fluorescent material as the controls

The injection of 10 µg histamine however resulted in a marked endoneurial exsudation of fluorescent products in all the nerves Fluorescence appeared not only the injection site but also around scattered endoneurial blood vessels along the whole sciatic nerve (Fig 6)

When EBA was injected 15 min after the local injection of 10 µg histamine there was only a slight local exsudation in two nerves while the other four appeared entirely normal

Rats treated with mepyramine maleate before the local injection of 10 µg histamine showed only a slight local exsudation of fluorescent products at the site of the injection in all the nerves



Fig 6 Microinjection of histamine into the endoneurium preceded by intravenous injection of EBA Intense fluorescence in and outside the walls of an endoneurial blood vessel



Fig 7 15 min after endoneurial injection of Compound 48/80 preceded by intravenous injection of EBA Fluorescence in the walls of an endoneurial blood vessel

Thus as for 5 hydroxytryptamine the permeability increasing capacity of histamine on endoneurial blood vessels was of early onset and brief duration This effect was abolished by pretreatment of the rats with the histamine antagonist, mepyramine maleate The different response between 5 hydroxytryptamine and histamine when the same dose was injected might reflect different permeability increasing activities of the two agents Previously, the permeability increasing activity of 5 hydroxytryptamine has been found to be more powerful than that of histamine on blood vessels of the skin and the muscle of the rat (Rowley & Benditt 1956 Majno & Palade 1961 Rowley 1964)

Compound 48/80

EBA administered to rats before the local injection of Compound 48/80 displayed intense red fluorescence 15 min later at the site of the injection both in and outside the walls of endoneurial blood vessels There was also an extravasation around several blood vessels all along the nerve (Fig 7) Two hours after the injections a diffuse extravascular red fluorescence was observed in the endoneurium of all injected nerves (Fig 8) The most intense fluorescence appeared between nerve fibres especially in the vicinity of endoneurial blood vessels



Fig 8 2 hrs after endoneurial injection of Compound 48/80 preceded by intravenous injection of EBA Note the diffuse rich extravascular fluorescence in the endoneurium

Rats injected with EBA 15 min and 2 hrs after the local injection of Compound 48/80 displayed fluorescence in and just outside scattered endo neurial vessels all along the nerve In all cases the amount of extravascular fluorescent material was markedly less than in the preceding groups (Fig 9)

The combined treatment with methysergide and mepyramine before the local injection of Compound 48/80 resulted 15 min later in a weak to moderate diffuse extravascular fluorescence at the site of the injection and a patchy occurrence of small fluorescent droplets in and just outside the walls of endoneurial blood vessels in the other parts of the nerves (Fig 10) 2 hrs after the local injection a diffuse faint extravascular fluorescence was observed throughout the nerves

Thus the local injections of Compound 48/80 were associated with a markedly increased permeability of endoneurial blood vessels with a rapid onset but which in contrast to the injections of either 5 hydroxytryp tamine or histamine still persisted 2 hrs after the injection Pretreatment of the rats with the 5 hydroxytryptamine antagonist methysergide and the histamine antagonist mepyramine markedly reduced but did not entirely inhibit the increased permeability

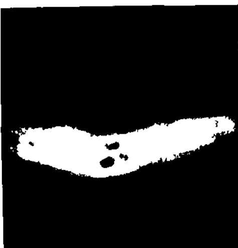


Fig 9 Intravenous injection of EBA 2 hrs after local injection of Compound 48/80 Fluorescence in the walls of an endoneurial blood vessel



Fig 10 Pretreatment with methysergide and mepyramine before injection of EBA and Compound 48/80 The rat was killed 15 min later Fluorescence in the walls of an endoneurial blood vessel

II Local application to the epineurium of exposed sciatic nerves

When EBA was injected prior to the local application of either histamine 5 hydroxytryptamine or Compound 48/80, a diffuse intense extravascular fluorescence was observed after only 5 min (Fig 11)

The fluorescent products extended to the innermost parts of the epineurium and perineurium leaving the underlying parts of the endoneurium devoid of extravascular fluorescence No difference in fluorescence intensity or extent of the fluorescent material could be detected between rats injected with histamine 5 hydroxytryptamine or Compound 48/80 On the control side of these rats numerous small fluorescent droplets and a faint diffuse extravascular fluorescence were observed in and outside the walls of epineurial and perineural blood vessels

15 min and 2 hrs after the local application of the permeability changing agents fluorescence in the epineurium and the perineurium was more intense than in the preceding group No difference could be observed between the experimental nerves of rats studied 15 min and 2 hrs after the local injections The control nerves of these rats displayed a diffuse faint extravascular fluorescence in the epineurium and the perineurium The intensity of the fluorescent light was most marked in rats studied 2 hrs



a



b



c



d

Fig 11 Intense diffuse fluorescence in the epineurium and the perineurium 5 min after local application of the permeability changing agents to the exposed sciatic nerve Intravenous injection of EBA prior to the local application Arrows marke the border between perineurium and endoneurium

a Histamine
b 5 hydroxytryptamine
c Compound 48/80
d Control (local application of Ringer solution)

after the local application of Ringer solution When EBA was injected 15 min after the local injection of permeability changing agents no difference

could be detected between experimental and control nerves in the intensity or the extent of the extravascular fluorescence

Pretreatment of rats with methysergide eliminated the difference between experimental and control side after the local injection of 5-hydroxytryptamine and Ringer solution respectively In a similar manner, mepyramine completely countered the difference observed after the injection of histamine and Ringer solution After the combined pretreatment of rats with methysergide and mepyramine of rats subjected to local injections of Compound 48/80 and Ringer solution, no difference could be detected in the fluorescent microscopic appearance of the epineurium and the perineurium in the two nerves

DISCUSSION

The capacity of 5 hydroxytryptamine to heighten vascular permeability has previously been investigated after local injection into skin (Rowley & Benditt 1956 Parrat & West 1957 Rowley 1964) or cremaster muscle of the rat (Majno & Palade 1961 Majno Palade & Schoefl 1961 The permeability increasing effect of this amine is generally thought to be of rapid onset and brief duration (Spector & Willoughby 1963 1964 1965) Studies on whole specimens of the cremaster muscle of rats using circulating carbon or mercuric sulfide for demonstration of vascular permeability, revealed that the part of the vascular tree affected by local injections of 5 hydroxytryptamine tended to be the venules rather than the capillaries (Majno Palade & Schoefl 1961) The detailed mechanism by which 5 hydroxytryptamine induces an increased permeability of blood vessels is not fully understood and the published results somewhat contradictory Thus recent electronmicroscopic studies have demonstrated that numerous leaks develop around the junctions between adjacent endothelial cells (Majno & Palade 1961) In contrast Alksne (1959) in a study on the effect of histamine on the passage of colloidal mercuric sulfide particles across dermal capillary walls considered that the passage took place across the endothelial cytoplasm rather than through intercellular spaces Both groups of investigators considered that the increased permeability was induced by the direct action of the amine on the endothelial cells Rowley (1964) however demonstrated that the increased permeability might in part be secondary to a constriction of veins through the action of 5 hydroxytryptamine

The present study shows that injections of 5 hydroxytryptamine into rat sciatic nerves are accompanied by the appearance of the tracer, EBA in and around vascular walls for a long distance along the endoneurium while control injections of Ringer solution only elicited slight local changes The exsudation of the tracer accompanying the local injection of 5 hydroxytryptamine was of brief duration and could be markedly reduced by pretreatment of the rats with the serotonin antagonist methysergide bimaleinate

The findings were similar in the epineurium after the local application of 5 hydroxytryptamine to the surface of exposed sciatic nerves These results strongly suggest that both endoneurial and epineurial blood vessels

SUMMARY

The effect of 5 hydroxytryptamine histamine and Compound 48/80 on the permeability of endoneurial and epineurial blood vessels in rat sciatic nerves was studied The agents were microinjected into the endoneurium or applied to the epineurium of exposed nerves Vascular permeability was demonstrated by fluorescent microscopic tracing of intravenously injected serum albumin labelled with Evans blue according to Steinwall and Klatzo (1965) The main findings were as follows

1 Injections of 5 hydroxytryptamine and histamine were associated with a marked increased permeability of endoneurial blood vessels of rapid onset and brief duration This effect was inhibited by pretreatment with a 5 hydroxytryptamine antagonist (methysergide bimaleinate) and a histamine antagonist (mepyramine maleate) respectively

2 Injections of Compound 48/80 were associated with a markedly increased permeability of endoneurial blood vessels with a rapid onset and lasting at least 2 hrs after the injection Preatreatment of rats with mepyramine and methysergide in combination markedly reduced but did not entirely inhibit the increased permeability

3 Applications of 5 hydroxytryptamine histamine and Compound 48/80 to the epineurium of exposed sciatic nerves were associated with a markedly increased permeability of epineurial blood vessels of rapid onset and brief duration Pretreatment of rats with mepyramine completely eliminated the action of histamine methysergide the action of 5 hydroxytryptamine and mepyramine and methysergide combined the effect of Compound 48/80

Thus the mast cell amines histamine and 5 hydroxytryptamine are capable of inducing increased permeability of blood vessels in rat peripheral nerves The permeability increasing capacity of Compound 48/80 seems to be mainly mediated by biogenic amines released from mast cells in the nerves

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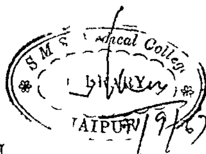
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STUDIES ON INTESTINAL DIPEPTIDASES

BY

TOR LINDBERG

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CONTENTS

This dissertation is based on the following publications

I Intestinal dipeptidases I Spectrophotometric determination and characterization of dipeptidase activity in pig intestinal mucosa (In collaboration with L Josefsson) *Biochim biophys Acta* (Amst) 1965 105 149–161

II Intestinal dipeptidases II Distribution of dipeptidase activity in the small intestine of the pig (In collaboration with L Josefsson) *Biochim biophys Acta* (Amst) 1965 105 162–166

III Intestinal dipeptidases III Characterization and determination of dipeptidase activity in adult rat intestinal mucosa (In collaboration with L Josefsson) *Acta physiol scand* 1966 *66* 410–418

IV Intestinal dipeptidases Characterization, development and distribution of intestinal dipeptidases of the human foetus *Clin Sci* 1966 30 505–515

V Intestinal dipeptidases Dipeptidase activity in the mucosa of the gastrointestinal tract of the adult human *Acta physiol scand* 1966 66 437–443

VI Intestinal dipeptidases Development of dipeptidase activity in the small intestine of the rat as related to the development of the intestinal mucosa (In collaboration with Ch Owman) *Acta physiol scand* 1966 In press

The papers are referred to in the text by their respective Roman numerals

In the gastrointestinal tract, the various proteolytic enzymes hydrolyze the dietary protein as well as the endogenous protein (enzymes, mucus dis rupted mucosal cells), which constitutes the major part of the protein in the intestinal lumen (for ref see Nasset 1964) Results from several investigations since those of van Slyke and Meyer 1912 and 1913 have given rise to the opinion that protein is completely hydrolyzed to its con stituent amino acids before leaving the small intestine Fisher in 1954 pointed out that no direct evidence supporting this theory had so far been presented However, later and more conclusive investigations (Leven son, Rosen and Upjohn 1959 Dawson and Holdsworth 1962, Dawson and Porter 1962) have corroborated the earlier opinion, and it is now widely accepted that by far the largest part of the protein enters the blood from the gut as free amino acids

Knowledge about the action of such enzymes as pepsin, trypsin, and chymotrypsin that take part in the initial hydrolysis of the protein is now well established (*cf* Bovey and Yanari 1960 Desnuelle 1960 a, b Hill 1965) The further hydrolysis of the protein is continued by the ac tion of the well characterized carboxypeptidases from pancreas and by leucine aminopeptidase from intestinal mucosa (*cf* Neurath 1960 Smith and Hill 1960) However there are in the intestinal mucosa, other en zymes associated with the final digestion of the protein These enzymes can be divided into two groups polypeptide splitting enzymes (Smith 1951) and dipeptide splitting enzymes

Several investigations during the past 60 years have demonstrated the dipeptide splitting activity of mucosal extract (see p 12–13) It has also been shown that this enzyme group acts only on dipeptides a free car boxyl group and a free amino group adjacent to the peptide bond is neces sary for its action (for ref see Smith 1951) These enzymes can therefore be classified as dipeptidases (dipeptide hydrolases EC 3 4 3) As far as is known tripeptide splitting enzymes have no or only very weak activity for dipeptides (Smith 1951, 1960) Concerning leucine aminopeptidase it has been demonstrated that purified preparations of this enzyme can

split some dipeptides, although at a much lower rate than their corresponding amide compounds (Smith and Hill 1960) The important role of the intestinal dipeptidases in the final digestion of the protein is apparent from the investigations of Agar, Hird and Sidhu (1953), Newey and Smyth (1959, 1960), and Wiggans and Johnston (1959), who showed that, *in vitro*, dipeptides (except for a small amount of glycylglycine) are hydrolyzed to their constituent amino acids before or during their transfer through the intestinal wall

Lack of methods suitable for assaying the activity in crude tissue extracts and the instability of the enzymes have hampered the studies on the intestinal dipeptidases In relation to other known proteolytic enzymes in the gut, relatively little information is available in the literature about the biochemical and physiological properties of this group of enzymes Moreover, there is no information about the occurrence and distribution of the intestinal dipeptidases in man thus, we know nothing about their significance in various pathological conditions of the gastrointestinal tract Results from recent studies on gluten induced enteropathy (Frazer 1962, Messer, Anderson and Hubbard 1964, Bronstein, Haeffner and Kowlessar 1966) suggest that this disease may be caused by a congenital defect of some peptidase(s) in the intestinal mucosa Before this can be definitely proved, however, it is necessary to get more information about the peptidases in the intestinal mucosa under physiological conditions

For this purpose, a study of dipeptidase activity in extracts of intestinal mucosa, from various aspects, was started in our laboratory, as a rapid and simple spectrophotometric assay method had been devised (Josefsson 1964, I) This method utilizes the strong absorption of the peptide bond in the low ultraviolet range By the use of ethanol with negligible absorption down to 210 mμ as precipitating agent, the method allowed determination of dipeptidase activity in crude tissue extracts

Our knowledge about the intestinal dipeptidases has been derived from investigations either of some single problems of the properties of the enzymes or of a single animal species The literature lacks collected information obtained by the same assay method concerning the intestinal dipeptidases The present study was therefore directed to a general survey of the main properties, of the distribution along the gut, and of the development of some dipeptidase activities in the intestinal mucosa Knowledge about the main properties of this enzyme group is important for further studies in this sphere By extending the study to three different species, we have obtained a comparative analysis of the biochemical and physiological properties of the enzymes

Earlier methods

The methods previously used in studies of dipeptidase activity are principally based on the determination of either the free carboxyl groups or the free amino groups formed when dipeptides are hydrolyzed Macro methods were in use during the first decades of this century e g the formoltitration method of Sørensen (1908) and the amino nitrogen determination of van Slyke (1912) These were unsuitable for large series of enzyme analysis A great improvement followed the introduction of micro methods such as that of Grassmann and Heyde (1929) (measurement of liberated carboxyl groups by titration with alcoholic KOH) and that of Linderstrøm Lang and Holter (1931) (titration of amino nitrogen with alcoholic HCl in acetone solution Linderstrøm Lang 1927) These two methods, as well as the ninhydrin method described by Moore and Stein (1948 1954) which, based on the colour reaction between ninhydrin and NH_2 groups determines the amino acids photometrically, are still extensively used in assays of dipeptidase activity in crude tissue extracts and partially purified preparations However, because these methods are non specific, they are adequate only when highly purified enzyme preparations are used Further, although they have been classified as micro methods they require a relatively large amount of substrates In 1938, Linderstrøm Lang and Lanz described a dilatometric micro method for peptidase activity, whose accuracy was about 50 times that of the earlier reported micro titrimetric method (Linderstrøm Lang and Holter 1931) This method, however proved too laborious to be used in routine analysis

Various modifications of the mentioned methods have also recently been used for assaying dipeptidase activity (see for example, Connell Dixon and Hanes 1955 Rademaker and Soons 1957, Glasser and Hanson 1962 Bryce and Rabin 1964 Matheson and Tattrie 1964)

Present method

Principle Dipeptides have been known to exhibit a strong absorption in the low ultraviolet range in contrast to most of the amino acids (Magill

Steiger and Allen 1937 Saidel, Goldfarb and Waldman 1952, Saidel 1955) This strong absorption is mainly due to the peptide bond, a condition that has been utilized for determining protein concentration (for ref see Wetlaufer 1962) The difference in absorption between dipeptides and amino acids offers a possibility, already pointed out by Saidel in 1955, for studying the dipeptide splitting activity, because the absorption will decrease as peptide bonds are split This condition was utilized by Binkley and Torres (1960) in assaying leucine aminopeptidase activity in partially purified preparations, and by Schmitt and Siebert (1961, 1964) who studied some dipeptidase activities in extracts from pig and rat kidney and from cod muscle However, as crude tissue extracts and partially purified preparations contain a relatively large amount of substances—for example proteins and carboxylic acids, which have a high absorption in the low ultraviolet range and thus interfere with the determination—this method has *a priori* the same limitations as the earlier ones mentioned above

For a spectrophotometric assay method to be useful, therefore these disturbing substances had to be eliminated Ethanol with negligible absorption down to 210 mμ proved suitable for this purpose it did not influence the differences in the absorption between the dipeptides and amino acids (I, III) This was demonstrated by comparing the spectra of the dipeptides and of their constituent amino acids—obtained in aqueous solution— with the corresponding spectra obtained from the digest mixture made according to the standard assay procedure (see below) and precipitated with ethanol at zero time and read against water It was also found that the absorbancy of the amino acids did not change during the incubation with active enzyme solution or with water For this reason any occurring digestion of the protein or polypeptides present in the enzyme solution did not influence the absorbancy Moreover, the absorbancy of the dipeptides was unchanged after incubation with inactivated enzyme solution or with water Therefore the decrease of the absorbancy obtained when dipeptides were incubated with enzyme preparation must be ascribed to the splitting of the peptide bonds in the dipeptides

When work is done in the low ultraviolet range, some instrumental limitations exist (Saidel Goldfarb and Kalt 1951 Tombs Souter and Maclagan 1959) The concentrations of the various dipeptides were thus chosen to give not too high absorbancies as otherwise the readings would be difficult and uncertain At these concentrations, suitable difference absorbancy of about 0 8 was found at the wavelength of 220 mμ At this wavelength the absorbancy of the blanks containing the amino acids was

low, allowing a narrow slit width of the spectrophotometer These procedures reduced the risk of interference from stray light occurring in the low ultraviolet range (for ref see above)

Procedure The standard assay procedure, described in detail in ref I is briefly outlined here

Aqueous solutions of the various dipeptides in appropriate concentrations served as substrates, aqueous solutions of the analogous amino acids in equimolar concentrations served as blanks

The enzyme solution was prepared at 4° Mucosa was scraped off the small intestine and homogenized in 0 1 M NaCl After standing for 30 minutes the homogenate was centrifuged at 4° for 30 minutes at 27 000 ×g The supernatant was used as enzyme solution after suitable dilution with water

20 μl of the enzyme solution was added to an equilibrated (40°) mixture containing 50 μl of dipeptide solution and 100 μl of buffer solution (0 15 M borate or phosphate buffer) and thoroughly mixed The incubation was performed at 40 and after suitable time 1 3 ml of ethanol water (99 1 v/v) was added and mixed with the digest mixture This procedure precipitated high molecular substances and interrupted the hydrolysis The obtained precipitate was centrifuged down (5000 r p m for 20 minutes) and the absorbancy of the supernatant was measured in a semi microcuvette at 220 mμ with a spectrophotometer

Samples containing the analogous amino acids, in equimolar concentration instead of dipeptides but treated identically in every other respect, were used as blanks

In a large number of estimates with the various enzyme concentrations used, it was invariably found that the absorbancy of the digest mixture containing amino acids did not change during incubation Because of this, in the later part of the study the time blanks were mostly eliminated, being included only at irregular intervals as controls and when a new source of enzyme preparation was used

Under the conditions used, we found a zero order reaction rate of the initial hydrolysis of the five dipeptides studied with extracts from pig rat, and human intestinal mucosa The hydrolysis of glycylglycine and glycyl L leucine followed a linear course until 30 % and 40 % respectively of the substrate were split whereas L alanyl L glutamic acid L alanyl-L proline, and glycyl L valine were hydrolyzed in a linear course until within 60-70 % of completion

By adding various concentrations of the enzyme solution, a linear cor

relation was found to exist between the rate of the hydrolysis of the various dipeptides and the enzyme concentrations used (within a 4 fold range)

General considerations on the assay procedure The present method is rapid and simple, it requires small amounts of substrate It has also a high sensitivity, allowing the hydrolysis of $5 \times 10^{-4} - 10^{-5}$ mmole of dipeptide to be measured, and a slight modification of the procedure allows further increase of the sensitivity, as recently shown in our laboratory (Josefsson, Norén and Sjostrom—in preparation) The incubation time can be varied up to about 80 minutes without influencing the linear course of the hydrolysis At longer incubation times, the linearity was lost probably because of inactivation of the enzyme Variations of the enzyme concentration did not influence the absorbancy at zero time Moreover, a variation of the amount of water in the incubation mixture within a range of ± 20 % had no effect on the absorbancy The slight influence of the buffer ions on the absorbancy is demonstrated by the finding that a variation of the concentration of the buffer salts within a range of ± 70 % did not change the absorbancy at zero time more than ± 10 % Increasing or decreasing the ethanol volume bv about 20 % changed the absorbancy less than 10 %

The accuracy of the method was found to be as high as ± 1 %—in experienced hands

These advantages of the method together with the fact that it permits assaying dipeptidase activity in crude tissue extracts not only from the intestinal mucosa but also from other sources (Josefsson, Noren and Sjostrom—in preparation), make the procedure very suitable for routine analysis

However, the method is limited in that hydrolysis of dipeptides containing aromatic amino acids cannot be measured, because these amino acids exhibit a high absorbancy in the low ultraviolet range (for ref see Wetlaufer 1962)

Routine control of the absorption of the various reagents used is necessary the addition of substances with a high absorption in the low ultra violet range may disturb the readings

Unit of dipeptidase activity

According to the recommendations of the Commission on Enzymes of the International Union of Biochemistry (1961) 1 unit is defined as the activity hydrolyzing 1 μmole of dipeptide per minute at 40°

When working with biological material, it is difficult to find a satisfactory reference substance to relate the enzyme activity to Theoretically, the use of the number of homogenated epithelial mucosal cells as a reference must be the one most free from objection However, that is impossible in this type of study It was decided therefore to relate the enzyme activity to the total amount of nitrogen in the added enzyme solution, determined by the micro Kjeldahl procedure The specific activity of the dipeptidases has thus been expressed as units per mg N present in the enzyme solution

PREVIOUS STUDIES ON INTESTINAL DIPEPTIDASES

Cohnheim demonstrated in 1901 that extract of intestinal mucosa decomposes pepton This proteolytic component of mucosal extract was called erepsin

A few years later, Abderhalden and Teruuchi (1906) and Euler (1907 a, b) reported that glycylglycine was split by the action of bovine and of pig intestinal mucosa The effect of pH on this reaction was shown by Dernby (1917), who found a pH optimum of about 7 8 for the glycyl glycine splitting activity of pig erepsin This finding was later confirmed by von Euler and Josephson (1926 a) In another report, von Euler and Josephson (1926 b) demonstrated that the glycylglycine splitting activity of pig erepsin was inhibited if the amino group of the dipeptide was benzoylated The authors concluded that a free amino group in the di peptide was essential for the enzyme action The problem of the specificity of the dipeptidase action was further studied by Bergmann and co workers Bergmann *et al* (1935) confirmed that the presence of a free amino group was necessary, they showed that the enzyme required also a free carboxyl group for its action This condition had earlier been found valid for yeast dipeptidase (Grassmann and Dyckerhoff 1928)

During the first decades after Cohnheim s discovery, the action of erepsin was generally believed to be due to a single enzyme The first attempts to fractionate erepsin were made by Waldschmidt Leitz, Balls and Waldschmidt Graser (1929) who reported that their pig intestinal extract consisted of a polypeptidase and a dipeptidase As substrates DL leucylglycylglycine and DL leucylglycine were used In 1929, Linder strøm Lang and Linderstrøm Lang and Sato demonstrated two different activities in pig intestinal mucosa a leucylpeptidase (substrate leucyl glycine) and a dipeptidase (substrate glycylglycine) indicated by different pH optima and stability These activities were reported to be distinct from the mentioned polypeptidase The leucylpeptidase was later called leucine aminopeptidase (Smith and Bergmann 1944) In 1936, Johnson Johnson and Peterson made the important observation that leucylpeptidase was activated by Mg^{2+} ions The effect of bivalent metal ions was further

studied by Berger and Johnson (1939), who reported that the hydrolysis of leucylglycine was activated by Mn^{+} ions to an even higher degree They also observed that Mn^{+} ions and, above all, Co^{2+} ions markedly accel erated the hydrolysis of glycylglycine The influence of various bivalent metal ions on dipeptidase activities in among other sources, extracts from rabbit and guinea pig intestine was further studied by Maschmann (1941 a, b) From the results of the studies on activation and specificity of various dipeptide splitting activities (Gailey and Johnson 1941), Johnson and Berger (1942) suggested that erepsin contained a number of dipeptidases Observations supporting this suggestion had earlier been reported by Grassmann von Schoenebeck and Auerbach (1932) who found that L prolylglycine was split by an enzyme prolinase in erepsin suggested as being different from other known enzymes and as acting differently Bergmann and Fruton (1937) observed that glycyl L proline was hydro lyzed by erepsin This enzyme activity, called prolidase was also sug gested as being distinct from other enzyme activities in erepsin Prolidase was also studied by Smith and Bergmann (1944) who found that the enzyme was influenced by bivalent metal ions, as were the other dipepti dases

Detailed studies of the glycylglycine and glycyl L leucine splitting activi ties in extracts from various tissues such as pig intestinal mucosa human uterus and rat muscle, were carried out by Smith who in 1951 summa rized the results in a review on the specificity of certain peptidases On the basis of these investigations he suggested (1948 a) that the glycyl glycine dipeptidase activity was extremely specific and later (1948 b) showed that a dipeptidase hydrolyzed glycyl L leucine since substituting the amino group or the carboxyl group in the dipeptide prevented enzyme action Various bivalent metal ions influenced specifically the glycyl L leucine dipeptidase

Intestinal dipeptidases have in recent years been studied by Robinson and Shaw (1960) Using rat intestinal mucosa they investigated the effect of pH and bivalent metal ions on the activities on L leucylglycine glycylglycine glycyl L leucine, and glycyl L alanine The distribution of the activities along the gut was also studied and the ileum was found to contain the highest activities Robinson (1960) demonstrated that glycyl glycine and glycyl L valine dipeptidase activities were low in the large intestine He studied the distribution of L leucylglycine and glycylglycine dipeptidase activities in subcellular fractions of the rat intestinal mucosa (1963) and found that 80–90 % of the activities were left in the super natant fraction

To summarize The previous investigations have demonstrated that the intestinal mucosa contains an unknown number of enzymes acting on dipeptides, that the enzymes hydrolyze the peptide bond of the dipeptide only if free terminal amino groups and carboxyl groups are present—classifying them as dipeptidases, and that bivalent metal ions influence the action of the enzymes Moreover, the general opinion is that the in testinal dipeptidases have a high specificity, although the enzymes have not yet been purified

Dipeptidase activities are found not only in the mucosa of the gastro intestinal tract, but have been demonstrated in all animal tissues hitherto examined, for example, liver, and kidney (e g Maschmann 1941 a, b, Abderhalden 1943 Rademaker and Soons 1957, Hanson and Blech 1959, Wilcox and Fried 1963, Vescia and Traniello 1964, Patterson *et al* 1965), uterus and muscle (Smith 1948 a, b) placenta (Vescia and Fidanza 1948, Zoch 1965), and the lense of the eye (Glasser and Hanson 1963) Erythro cytes, leukocytes lymphocytes, and serum contain dipeptidase activities (e g Adams, McFadden and Smith 1952, Fleisher 1953, 1955, Haschen 1961 1963), which have also been found in micro organisms and plants (for ref see Johnson and Berger 1942) In fact most of the investigations on dipeptidases reported so far have been performed, especially in recent years, on extracts from tissues other than the intestine Although Smith (1951) observed a difference concerning the stability and the influence of bivalent metal ions of glycylglycine and glycyl L leucine dipeptidase from various tissues, it has not yet been proved that the activity from various tissues against the same dipeptide is related to different enzyme molecules

PRESENT INVESTIGATION ON INTESTINAL DIPEPTIDASES

The investigation was made on pig rat, and man The pig which nutritionally resembles man was chosen because large amounts of mucosa can easily be obtained Thus, the intestinal mucosa of this animal is suitable for purification studies that require large amounts of enzyme material The rat is a convenient laboratory animal and has been extensively used in intestinal absorption studies Clinically knowledge of the various properties of the intestinal dipeptidases in man is of great importance especially as it has now been possible through the development of biopsy technique to study the function of the intestinal mucosa in various pathological conditions

When selecting substrates from the large number of naturally occurring dipeptides we considered three different aspects the dipeptides would theoretically be split specifically they should be easily obtainable commercially and finally the activities on some of the dipeptides should have been investigated earlier On these grounds we chose the following five dipeptides L alanyl L glutamic acid containing the acidic amino acid glutamic acid L alanyl L proline an imidodipeptide glycylglycine the most simple dipeptide, whose hydrolysis has been studied before and is suggested to be very specific (see p 13) glycyl L-leucine whose hydrolysis has also been studied before (se p 13) and glycyl L valine whose structure differs little from glycyl L leucine This dipeptide was included in order to study whether any resemblance existed between the activities on these two substrates

The present study can be divided into three parts
1) general characteristics of intestinal dipeptidases
2) distribution along the gut and specific activity of intestinal dipeptidases
3) development in the prenatal life of intestinal dipeptidases

The results are given in the following paragraphs

General characteristics of intestinal dipeptidases

The results reported below are based on experiments performed on at least three different intestines from each species

Table I *Comparison of pH optima for dipeptidase activities in pig rat and human intestinal mucosa*

Substrate	Pig	Rat		Man	
		Foetus	Adult	Foetus	Adult
L-Alanyl L-glutamic acid	7 4	7 2	7 3	7 5	7 3
L-Alanyl L-proline		7 0	7 0	7 0	7 0
Glycylglycine	7 9	7 5[1]	7 5[1]	7 6	7 8
Glycyl L-leucine	7 9	7 4	7 5	7 9	7 6
Glycyl L-valine	7 6	7 5	7 4	7 8	7 6

In the presence of Co^{+} ions

Effect of pH Various phosphate and borate buffer solutions were used for studying the influence of pH on the dipeptidase activities The pH values in the digest mixture (measured by glass electrode) before and after digestion were found to differ less than 0 1, which agrees with the observation of Smith (1948 a) when studying glycylglycine dipeptidase When determining the pH optima, we chose the initial pH values in the digest mixtures Table I records a comparison of the obtained pH optima for the various activities in the different species including rat and human foetus (I III IV V VI) As seen from the table, the pH optima for the various dipeptide splitting activities varied between 7 0 and 7 9 Some smaller differences of the pH optima between the various species are also evident

In the literature reports are available of the influence of pH on the activities on glycylglycine glycyl L leucine and glycyl L valine Dernby (1917) found in extracts from pig intestinal mucosa the pH optimum for *glycylglycine* dipeptidase activity to be 7 8 von Euler and Josephson (1926 a) found it to be 8 0 whereas Linderstrom Lang and Sato (1929) reported it to be 7 3 Robinson and Shaw (1960) observed in rat intestinal mucosa two pH optima 7 4 and 8 2, which finding together with an observed difference of the effect of Mn^{+} ions, was interpreted as indicating that two enzymes were present

Studying rat liver glycylglycine dipeptidase Wilcox and Fried (1963) also found among other variables studied two pH optima 8 1–8 2 in the residual system and 8 5 in the Co^{+} ion or Mn^{+} ion system From other tissues, such as human uterus (Smith 1948 a pH optimum 7 6) human erythrocytes (Haschen 1963 pH optimum 8 0) and human leucocytes

(Fleisher 1955: pH optimum 7.7), only one pH optimum was observed, whereas Fleisher (1953) studying normal serum found three optima in the Co^{2+} ion system (6.2–6.3, 6.8, and 8.0–8.3) but only one optimum in the Mn^{2+} ion system (7.4). In the present study, only one pH optimum for glycylglycine dipeptidase activity in the three species investigated was observed in the Co^{2+} ion system or in the residual system (i.e. no metal ions added to the crude tissue extract).

Investigating *glycyl-L-leucine* dipeptidase activity in rat intestinal mucosa Robinson and Shaw (1960) found two pH optima (7.6 and 8.6). This could not be confirmed in the present study, but in rat, the activity declined slowly in the pH range between 7.5 and 8.5.

In extracts from other tissues, such as bovine lense of the eye (Glässer and Hanson 1963: pH optimum 7.5), human erythrocytes (Haschen 1961: pH optimum 8.2) and normal serum (Fleisher 1953: pH optimum 9.4), only one pH optimum was observed.

Robinson (1960) reported the pH optimum of *glycyl-L-valine* dipeptidase activity in rat intestinal mucosa to be 8.4: this value differs distinctly from that observed in the present study.

It has been observed that the rate of the hydrolysis of glycyl-L-leucine is accelerated when phosphate buffer is used (Smith 1948 b, Haschen 1961, Glässer and Hanson 1963), probably owing to the binding of the inhibitory Ca^{2+} ions to phosphate (Smith 1948 b). At a comparison of the rate of the hydrolysis of the various dipeptides in the phosphate buffer system with that of the borate buffer system at the same final pH, no differences were found in the present study.

Influence of bivalent metal ions. As the dipeptidases are influenced by bivalent metal ions (Smith 1951), the effect of Co^{2+}, Mn^{2+}, Mg^{2+}, and Zn^{2+} ions on the hydrolysis was studied in the various species (I, III, IV, V and VI). From Table II, which gives a summary of the results, it is apparent that the pattern of the effect of the four metal ions on the hydrolysis differed from one dipeptide to another. However, the difference between glycyl-L-leucine dipeptidase activity and glycyl-L-valine dipeptidase activity was not so marked. At a comparison of the influence of the bivalent metal ions on the activities in the various species, it should be observed that for glycylglycine, glycyl-L-leucine and glycyl-L-valine dipeptidase activities different buffer systems were used in some of the species. It has been shown that the anions in the buffer system affect the influence of mainly the Zn^{2+} ions (Smith 1948 b, I, III). In the phosphate buffer system, the inhibitory effect of the Zn^{2+} ions was less than in the

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1955 Togasawa and Katsumata 1956 Rademaker and Soons 1957, Robin son and Shaw 1960 Haschen 1963, Wilcox and Fried 1963)

Concerning the *glycyl L leucine* dipeptidase activity, the inhibitory effect of Co^{+} ions and Mn^{+} ions has previously been reported in the literature (Maschmann 1941 b Smith 1948 b, Robinson and Shaw 1960, Haschen 1961) However, Smith (1948 b) reported that after dialyzing and filtering enzyme solution obtained from pig intestinal mucosa the activity was specifically activated by Mn^{+} ions In crude extracts of intestinal mucosa, Smith (1948 b) and Robinson and Shaw (1960) observed that the Mg^{+} ions also inhibited the reaction this could not be confirmed in the present study The inhibitory effect of Zn^{+} ions on the hydrolysis of glycyl L-leucine has previously been observed in studies on intestinal mucosa (Smith 1948 b, Robinson and Shaw 1960) as well as on other tissues (Fleisher 1953 Glasser and Hanson 1963) Nevertheless, this activity in human uterus and rat muscle is activated by these ions in the presence of phosphate buffer (Smith 1948 b)

Concerning the other dipeptidase activities studied no earlier data about the influence of bivalent metal ions have been found in the literature

When the significance of the obtained findings concerning the influence of the bivalent metal ions on the various activities is considered, it must be remembered that the experiments were performed on crude tissue extracts which from the beginning contain an unknown concentration of these ions If we add the various bivalent metal ions in varying concentra tions their influence on the reaction might however, be satisfactorily studied in spite of our lack of knowledge about their true concentration in the digest mixture Little is known about the activity of the intestinal dipeptidases where metal ions are not present Josefsson and Sjostrom (1966) have shown that when the mucosa is homogenated in 0 005 M EDTA the activity of glycyl L leucine dipeptidase is about one third of that when the mucosa is homogenated in 0 1 M NaCl

Distribution along the gastrointestinal tract and specific activity of intestinal dipeptidases

Distribution of dipeptidase activity along the gastrointestinal tract Various regions of the gastrointestinal tract are known to differ in their capacity to absorb the various nutrients By labelling the dietary protein its absorp tion in man has been shown to occur in the upper part of the small in testine shortly after ingestion (Borgstrom *et al* 1957, Crane and Neuber

ger 1960) However, so far, there has been no direct study on the dipeptide splitting capacity of the various regions of the human gut The only studies of this problem are those of Robinson and Shaw (1960) and Robinson (1960), who investigated the distribution of some dipeptidase activities in the rat intestine

The dipeptide splitting capacity was therefore determined in various levels of pig, rat, and human gastrointestinal tract (II, III, IV, V, VI) In order to compare the activities in the various sections examined the specific activities of the dipeptidases were calculated (units of dipeptidase activity per mg total nitrogen present in the enzyme solution)

The results showed that in pig (II) and adult man (V) the activities against the five dipeptides studied were very low in the proximal part of the duodenum In its distal part, the activities rose rather sharply and reached in pig, a maximum level in the jejunum and in the proximal part of the ileum In adult man, the activities were even higher in the terminal part of the ileum, whereas in pig the activities declined in this region In rat (III), however, the activities were high, even in the proximal part of duodenum Maximum values were found in the jejunum and in the upper part of the ileum in three of the five intestines studied These findings agree with those of Robinson and Shaw (1960) However, no significant difference between the various regions of the rat intestine was found in the present study In fact, in two of the intestines the activities were as high, or even higher in the duodenum as in the more distal parts of the gut

The amount of dipeptidase activity in various parts of the developing rat small intestine was determined in connection with the study of the development of rat intestinal dipeptidases (VI) Owing to the small size of the gut in this early age proportionally larger sections had to be taken for the preparation of enzyme solution From the 19 day stage *post coitum* up to and including birth the activity on L alanyl L proline was the same in the proximal third as in the middle and distal thirds whereas the activities on the other four dipeptides were higher in the distal two thirds of the small intestine In the postnatal stages up to the 3rd week *post partum* all the activities were slightly stronger in the middle or distal thirds of the small intestine

As fresh material is needed for the enzyme studies and as the investigation on adult man was performed on operation specimens it was impossible to determine the distribution of the activities along the whole length of the intestine in the same individual However, this was possible in the studies of the development of the enzyme activities in the human prenatal

life (IV) In human foetuses between 11 and 23 weeks of fertilization age, maximum dipeptidase activities were found in the proximal part of the gut, not in the middle and distal parts Although this difference was not significant, it seems that the distribution of the activities change during the later period of development It should be emphasized that proportionately larger sections of the human foetus intestine were investigated However, the same high activities were found in the proximal part of the gut even when examining sections as small as four per cent of the total length of the small intestine

A comparison of the distribution of the various dipeptidase activities revealed no significant difference In pig and rat, the level of maximum glycylglycine dipeptidase activity tended to be proximally localized compared with the level of the other activities

When we relate the findings concerning the distribution of dipeptidase activities in the human small intestine to our knowledge about the localization of the digestion and absorption of dietary protein over the intestinal length in man (Borgstrom *et al* 1957 Crane and Neuberger 1960) it is apparent that the activities in the distal duodenum and the upper jejunum are adequate for accomplishing the final digestion of the given protein The physiological role of the large amount of dipeptidase activity found in the distal jejunum and in the ileum is probably that of accomplishing the final digestion of the endogenous protein which as known (for ref see Nasset 1964) forms by far the largest part of the protein in the lumen and which is hydrolyzed and absorbed in the distal parts of the small intestine

The dipeptidase activities were determined also in the gastric and the large intestinal mucosa in rat (III), human foetus (IV), and adult man (V) The activities in the stomach were generally low compared with those found in the jejunum ileum Equally low activities were found in the large intestinal mucosa of rat and adult man The large intestine of human foetuses however contained the same amount of dipeptidase activities as the foetal small intestine The obtained difference between the specific activities in the adult and the foetal large intestine was significant for the activities on L alanyl L proline ($p < 0\ 005$) glycyl L leucine ($p < 0\ 05$), and glycyl L valine ($p < 0\ 05$) but not significant for L alanyl L glutamic acid dipeptidase activity ($p < 0\ 4$) as calculated according to Student s t test (The values for glycylglycine dipeptidase activity could not be compared since, in human foetus, this activity was measured only in the presence of Co^{+} ions) It should be pointed out that in adults only the mucosa was used as a source of the enzyme whereas in foetuses the

Table III *Comparison of specific activity (units per mg nitrogen, mean values ± SD) of intestinal dipeptidases from various species Enzyme solution obtained from jejunum Optimum pH*

Substrate	Pig	Rat	Adult man[1]
L-Alanyl L-glutamic acid	35 4 ± 7 7 (n=6)	10 8 ± 3 8 (n=10)	26 3 ± 5 4 (n=3)
L-Alanyl L-proline		7 95 ± 0 83 (n=5)	6 09 ± 3 34 (n=2)
Glycylglycine[2]	6 61 ± 2 94 (n=11)	1 30 ± 0 60 (n=7)	8 86 ± 1 95 (n=3)
Glycyl L-leucine	129 2 ± 24 8 (n=7)	26 1 ± 6 4 (n=11)	232 3 ± 57 7 (n=3)
Glycyl L-valine	81 4 ± 32 5 (n=8)	21 5 ± 7 1 (n=10)	111 4 ± 13 6 (n=3)

[1] Values obtained from the three patients EA EA and EA (V)
[2] No metal ions added

entire intestinal wall was used Thus, the obtained difference of the specific activities (units per mg nitrogen) are perhaps even greater The cause of this decrease of the activities in the large intestinal mucosa from foetal to adult life is difficult to establish An important difference between the adult and the foetal large intestine is the rich bacterial flora in the adult large intestine It is perhaps possible that some proteolytic enzymes pro duced by micro organisms in the large intestine destroy the intestinal hydrolases and thereby reduce their activity However it should also be noted that villi like projections are found in the large intestine in the foetal life period (Johnson 1913 Patzelt 1931, IV) and that these villi like structures disappear during the later foetal period To discover whether this disappearance is related to the decrease of the dipeptidase activities, it should be of interest to investigate intestines from newborn infants but it has so far been impossible to get fresh material from this age

Specific activities of intestinal dipeptidases in the various species To get an idea of the ability of the intestinal mucosa from the various species to split the dipeptides the specific activities of the dipeptidases in jejunum were calculated Table III presents a comparison of the values obtained (mean values ± SD) for the various activities in the different species If the various dipeptide splitting activities are first compared with one an other, it is evident that the activities on glycylglycine and L alanyl L proline were low compared with the high activities on glycyl L leucine and glycyl L valine The jejunum of pig and adult man contained about the same amount of activities with the exception of glycyl L leucine di peptidase activity which was highest in adult man In rat relatively low

Table IV *Comparison of relative specific activity (related to glycylglycine dipeptidase activity) of intestinal dipeptidases from various species Enzyme solution obtained from jejunum Optimum pH*

Substrate	Pig	Rat	Adult man[1]
L Alanyl L-glutamic acid	5 36	8 31	2 97
L-Alanyl L-proline		6 12	0 69
Glycylglycine[2]	1 0	1 0	1 0
Glycyl L-leucine	19 5	20 1	26 2
Glycyl L-valine	12 3	16 5	12 6

[1] Values obtained from the three patients EA EA and EA (V)
[2] No metal ions added

specific activities were found for four of the dipeptidases, whereas the fifth activity, L alanyl L proline dipeptidase was proportionately very high This became more obvious when the relative specific activities were calculated (Table IV)

As little is known about the occurrence and concentrations of the various dipeptides in the dietary and the endogenous protein it is at present impossible to relate to the physiological need the obtained findings concerning the capacity of the mucosa to split the various dipeptides

Prenatal development of intestinal dipeptidases

It has long been established that the newborn infant has no difficulty in digesting and absorbing protein (cf Smith 1959) The various proteolytic enzymes dealing with the digestion of the protein therefore appear to be present in sufficient amounts at birth Previous investigations on the development of proteolytic enzymes in man have concerned pepsin (e g Keene and Hewer 1929 Werner 1948, Wagner 1958), trypsin (e g Ibrahim 1909 Keene and Hewer 1929 Werner 1948), and erepsin (Jaeggy 1907, Langstein and Soldin 1908, Tachibana 1927 Keene and Hewer 1929) The proteolytic activity of erepsin was estimated by the biuret method which does not permit a determination of the breakdown of dipeptides In a short communication, Blum Jarmoschkewitsch and Jakowtschuk (1936) reported that intestinal glycylglycine dipeptidase activity assayed by the micro titration method could be demonstrated as early as in the second month of development of the human foetus However so far no detailed investigation into the prenatal development of the

Table V *Comparison of specific activity (units/mg nitrogen mean values ± SD) of intestinal dipeptidases in human foetus (11-–3 ueeks of fertilization age) and adult man Enzyme solution obtained from jejunum Optimum pH*

Substrate	Human foetus		Adult man[2]
	Entire intestinal wall	Mucosa (corrected mean values[1])	
L-Alanyl L-glutamic acid	18 8 ± 9 8 (n=23)	26 3	26 3 ± 5 4 (n=3)
L-Alanyl L-proline	4 80 ± 2 12 (n=19)	5 28	6 09 ± 3 34 (n=2)
Clycylglycine[3]	5 52 ± 1 00 (n=4)	6 62	8 86 ± 1 95 (n=3)
Glycyl L-leucine	139 5 ± 57 0 (n=25)	167 4	232 3 ± 57 7 (n=3)
Glycyl L-valine	81 4 ± 36 7 (n=21)	122 1	111.4 ± 13 6 (n=3)

For explanation see text
Values obtained from the three patients EA EA and EA (V)
No metal ions added

intestinal dipeptidase activities in any species has been reported in the literature

Dipeptidase activity in human foetus (IV) The study included 35 foetuses obtained at legal abortions performed by hysterotomy The fertilization age of the foetuses, determined by measuring the crown rump length (C R length) varied from 11 to 23 weeks (corresponding to 6 2 cm to 21 5 cm C R length Patten 1953) The dipeptidase activities were assayed in a section from the upper part of the small intestine (in foetuses smaller than 10 cm C R length the proximal third or fourth of the gut in larger foetuses in a 5 cm piece 10 cm from the pylorus) When the obtained specific activities of the various dipeptidases were plotted against the C-R length of the various foetuses, no significant difference was found between the values from the smallest compared with those from the largest Thus in this period of foetal life no increase of the dipeptidase activities occurred

The separate values of the various specific activities were therefore combined and the mean values ± SD were calculated and compared (Table V) with those obtained from three adult jejunal mucosal samples As the table shows the values of glycyl L leucine and glycylglycine dipeptidase activities in adult mucosa were about 60 per cent higher whereas the other activities were only slightly higher However as mentioned earlier because the foetal intestine was too thin to permit removal of the

mucosa in most of the foetuses, the entire intestinal wall was used for the enzyme preparation, whereas in the adult, only the mucosa was used This difference might be partly responsible for the higher values in adults Therefore, to test this, the dipeptidase activities in the mucosal samples and in samples from a consecutive section of the entire intestinal wall were assayed in some of the larger foetuses, where the mucosa could be scraped off, and the following relations between mucosal activities and whole wall activities were obtained for L alanyl L glutamic acid 1 4, for L alanyl L proline 1 1, for glycylglycine 1 2 for glycyl L leucine 1 2, and for glycyl L-valine 1 5 With the aid of these figures, the specific activities in the foetal mucosa were calculated, the results are given in Table V The activities on glycylglycine and glycyl L leucine remained somewhat lower in the foetuses but the difference is not significant It can therefore be concluded that the dipeptidase activities are fully developed at the foetal age of 11 weeks

Microscopical examinations of the structure of the mucosa in the actual foetal period revealed that it was well developed at this stage which agrees with the findings of Johnson (1910) and Patzelt (1931)

Dipeptidase activity in the prenatal and postnatal life of rat (VI) In the study of the development of the dipeptidase activities in human foetuses, it was possible to get fresh material only from foetuses of 11 to 23 weeks fertilization age In order to get an opportunity to study the development of the activities over a relatively longer period of foetal life rat foetuses were investigated

Three or four litters a day from and including day 15 *post coitum* up to birth (22–22 1/2 days *post coitum*), were investigated a total of 252 foetuses from 35 albino rats The following postnatal stages were also included newborn nonsuckled rats (6 litters), newborn suckled rats (about 6 hours of age, 3 litters) and finally rats from the third day and the second and third weeks of life (4 litters) Parallel with the enzyme analyses microscopical examination of the mucosal structure was made in all the stages

The investigation showed that in the period of mucosal cell proliferation (15 to 18 days *post coitum*) the dipeptidase activities were very low Between the 18 1/2 day stage *post coitum* and parturition, the activities increased uniformly and reached maximum values in the 22 day stage or in the newborn nonsuckled rats This increase coincides with the differentiating period of the mucosa But it is impossible to determine from this study what structural change in the mucosal cells is responsible for this

increase in the functional capacity of the intestine From a morphological aspect, it is interesting to note that the brush border membrane appears in the 18 1/2 day stage *post coitum* and that the anlage of the crypts of Lieberkuhn is not observed until the activities become maximum

The specific activities in newborn nonsuckled rats were about three times as high as those in adult rat However, the activities on L alanyl L glutamic acid, glycylglycine glycyl L leucine and glycyl L valine fell significantly to the relatively low adult level only a few hours after birth i e when the rats had sucked The activity on L alanyl L proline, on the other hand, declined successively in the postnatal period and first reached the adult level at the third week *post partum* The cause of the abrupt decrease of the four activities is at present not clear In the newborn period, several events occur feeding, bacterial invasion, hormonal adjustment etc The effect of rat colostrum on the dipeptidase activities was studied in some experiments It was found that the activity on L alanyl L proline was slightly inhibited, whereas the activities on the other dipeptides were considerably reduced The nature of the inhibitory factor(s) in colostrum is unknown and requires further studies The effect of bacterial invasion on the intestinal hydrolases has been studied by Dahlqvist, Bull and Gustafsson (1965) They found no difference between the disaccharidases in the small intestine of conventional adult rats and those in the intestine of germ free adult rats whereas large differences of some activities were observed in the caecum and the large intestine A study of the possible hormonal influence upon the enzyme activities has shown that corticosteroids can affect the development curves of the alkaline phosphatases (Moog 1962 Halliday 1959) and of the disaccharidases (Doell and Kretchmer 1964 Koldovsky and Chytil 1965)

At a comparison of the development of intestinal hydrolases in various species it must be remembered that the stage of the structural development of the mucosa at birth differs in various species Thus in the present study, the structure of the mucosa in newborn rats was found to be comparable with that of human foetus of about 12 weeks fertilization age In human foetuses the differentiating period of the mucosa has been shown to occur between 18 mm and 55-60 mm C R length (Johnson 1910, Patzelt 1931) thus just prior to the foetal period actually studied As the dipeptidase activities were well developed in the smallest foetuses investigated (62 mm C R length) and in view of the results from the study of developing rats it seems reasonable to suggest that the development of the dipeptidase activities also in man coincides with the differentiating period of the mucosa

GENERAL DISCUSSION

Specificity of intestinal dipeptidases Although it is important to determine whether the various dipeptide splitting activities are related to different specific enzymes or to the action of a single enzyme, no extensive purification studies of the intestinal dipeptidases have so far been reported Such studies have mostly been hampered by the lability of the enzyme activities The different effects of pH and bivalent metal ions on the various dipeptidase activities observed in this investigation, as well as in others are not of course, direct proof that the activities are related to different enzyme molecules

In a study on an approximately 2000 fold purified peptidase from swine kidney Vescia and Traniello (1964) found that this enzyme pre paration, which was not homogeneous split L leucylglycine in the presence of Mn^{+} ions glycylglycine in the presence of Co^{+} ions, and glycyl L-leucine in the absence of metal ions However it was suggested that the different activities were related to a single enzyme, as no difference was found in their relative rates of hydrolysis during the purification procedure, in their pH optima and pH stability, and finally in their in activation course Patterson *et al* (1965), studying rat liver peptidases, could separate two enzymes with glycyl L leucine splitting activity, glycylleucinase (S) and glycylleucinase (F) , but they were unable to distinguish L alanylglycine dipeptidase from glycylleucinase (F) However on the basis of the different shapes of the peaks of the enzyme activities after electrophoretic separation it was presumed that these two activities also were related to different enzymes Recently Campbell *et al* (1966) purified a particulate dipeptidase from hog kidney The purified fraction, which they found to be homogeneous in ultracentrifugation and in acrylamide gel electrophoresis experiments catalysed the hydrolysis of several dipeptides but did not act on tripeptides and L leucineamide

In the studies on the intestinal dipeptidases we have repeatedly ob served a different behaviour of especially L alanyl L proline dipeptidase activity (e g influence of pH metal ions and colostrum on the reaction the disappearance of this activity in meconium from newborn infants (IV)

and the relatively high activity in intestinal contents (Josefsson, Lindberg and Öjesjö—in preparation)) which argues in favour of the suggestion that this dipeptide is split by a separate enzyme It has recently been consistently demonstrated in purification studies on pig intestinal dipeptidases (Josefsson, to be published) that the activity on L alanyl L proline can be completely separated from the activities on L alanyl L glutamic acid, glycyl L leucine, and L-glutamyl L valine

Relation to other intestinal hydrolases Concerning the distribution of the intestinal hydrolases along the gut, it is of interest to note that in man the distribution of the dipeptidase activities is similar to that of the disaccharidases Thus, Auricchio *et al* (1963) reported that in man the activities of the intestinal disaccharidases maltase, invertase, isomaltase and lactase were low in the proximal part of the duodenum whereas the jejunum and the ileum contained large amounts of the activities The findings on pig in the present study resembled those of Dahlqvist (1961), who found in an adult pig low invertase, isomaltase, and maltase activities in the proximal duodenum, whereas they were high in the jejunum ileum region However, cellobias, lactase, and trehalase activities were strongest in the proximal part of the gut In rat (Dahlqvist 1963), invertase, isomaltase, and trehalase activities were highest in the proximal two thirds of the small intestine whereas maltase activity was equally high throughout the entire length of the gut, and lactase activity highest in the middle third (Dahlqvist and Thomson 1964)

The above mentioned investigations on pig and rat suggest that the various disaccharidases differ in their distribution even in the same species in contrast to the condition found valid for the dipeptidase activities

Regarding the development of the intestinal hydrolases, the disaccharidases appear early in human foetal life (Auricchio Rubino and Murset 1965 Dahlqvist and Lindberg 1966) as do the dipeptidases Dahlqvist and Lindberg (1966) studied the development of disaccharidase and alkaline phosphatase activities in the same human foetal material as used for the study of the development of the dipeptidase activities (IV) Invertase isomaltase, and maltase were found to be well developed already at the foetal age of 11 weeks whereas trehalase developed from the 11th to the 23rd weeks and lactase even later in the foetal life Although alkaline phosphatase activity increased from the 11th to the 23rd weeks of foetal life this activity was still below the adult level

When the present findings on developing rats are related to earlier studies on the development of rat intestinal disaccharidases, the prenatal

developing curve for lactase activity (Alvarez and Sas 1961, Doell and Kretchmer 1962) is found to resemble that of the dipeptidase activities In the postnatal period, the lactase activity successively decreases and reaches the low adult level in the fourth week *post partum* (Alvarez and Sas 1961, Rubino Zimbalatti and Auricchio 1964, Doell and Kretchmer 1962) The α disaccharidases, however are low or absent in the first two weeks *post partum* and thereafter increase rapidly to the adult level (Doell and Kretchmer 1963 1964 Rubino *et al* 1964)

Concluding remarks

The results from the present investigation have provided a basis for further studies on various problems concerning the intestinal dipeptidases, such as their clinical significance and the location of their physiological action

By the peroral small intestinal biopsy technique a sufficient amount of mucosa is obtained for assaying dipeptidase activity and as the spectro photometric assay method is suitable for routine analysis we are now able to make a more general study of the intestinal dipeptidases in relation to various disorders of the gut (Josefsson Lindberg and Norden 1966) Indirect evidence suggests that gluten induced enteropathy is perhaps due to a deficiency of one or more peptidases in the intestinal mucosa (Frazer 1962, Messer, Anderson and Hubbard 1964 Bronstein Haeffner and Kowlessar 1966) Attempts by Messer Anderson and Townley (1961) to demonstrate a deficient peptidase activity in duodenal biopsy specimens from coeliac children failed however although the specimens were reported as showing gross histological changes It is of interest therefore to note that of the results hitherto achieved in our investigation (Josefsson Lindberg and Norden 1966) the dipeptidase activities in the mucosa taken from the region of flexura duodeno jejunalis in patients with gluten induced enteropathy were significantly lower than those in a control material At an extension of the investigation to children, the same results were obtained (Lindberg unpublished observations) Whether the low intestinal dipeptidase activities observed in the patients with gluten induced enteropathy are only a secondary phenomenon due to the atrophy of villi in these patients or whether they are a specific feature of this disease remains to be established

The location of the physiological action of intestinal dipeptidases is still unsolved In fact the various investigations directed on this problem have produced various results supporting the theory that the hydrolysis

occurs mainly in the lumen of the gut (Szafran, Szafran and Oleksy 1962), on the external surface of the mucosal cell membrane (Ugolev *et al* 1964 Ugolev 1965), just before entering the mucosal cell or very rapidly within the cell (Dawson and Holdsworth 1962), and finally, within the mucosal cell (Newey and Smyth 1960, 1962) We are now studying this problem in our laboratory Results hitherto obtained show that intestinal content collected from man and rat contains only small amounts of dipeptidase activities, except for L-alanyl L proline dipeptidase activity (Josefsson, Lindberg and Öjesjo—in preparation) These results suggest that the hydrolysis of dipeptides occurring in the intestinal lumen is of minor importance in the final digestion of protein, with the possible exception of L alanyl L proline However it remains to be established whether the dipeptides are hydrolyzed outside the mucosal cell membrane or absorbed and split intracellulary

SUMMARY

A rapid and simple spectrophotometric assay method for assaying dipeptidase activity in crude extracts of intestinal mucosa is described By this method, the activities on five dipeptides L alanyl L glutamic acid, L alanyl L proline glycylglycine glycyl L leucine and glycyl L valine, in *the mucosa of the gastrointestinal tract of pig, rat* and man were determined

The influence of pH on the various activities was studied and the pH optima were found to vary from 7 0 to 7 9 The pH optimum for each dipeptide splitting activity differed slightly from one species to another

The rate of the hydrolysis of the dipeptides, except L alanyl L proline was generally influenced in all the species by the bivalent metal ions Co^{+} Mn^{2+}, and Zn^{+}, whereas the Mg^{+} ions generally did not affect the dipeptide splitting activities It was also found that the influence pattern of the bivalent metal ions on the hydrolysis differed from one dipeptide to another

The distribution of the dipeptidase activities along the gastrointestinal tract of pig rat foetus, adult rat human foetus and adult man was investigated Generally low activities were found in the stomach The jejunum and the ileum of all the species contained large amounts of dipeptidase activities In the proximal duodenum of pig and adult man, low activities were found in contrast to those found in adult rat and human foetus The large intestine in adult rat and adult man contained small amounts of activities whereas in that of the human foetus they were as high as in the small intestine and significantly higher than those in the adult large intestine

With one exception the *specific activities of the intestinal dipeptidases* were of about the same magnitude in the pig as in the adult human jejunal mucosa, the glycyl L leucine dipeptidase activity was strongest in adult man The rat jejunum however had relatively low specific activities except for L alanyl L proline dipeptidase, which was even higher in rat than it was in adult man

Studies on the development of the intestinal dipeptidases revealed that

in man the activities were fully developed at 11 weeks of fertilization age, i e in the smallest foetus studied The structure of the mucosa at this stage was well developed The study of developing rat revealed that the dipeptidase activities increased in the differentiating period of the mucosal cells Maximum activities were found at the parturition period Post natally, four of the activities fell abruptly to the relatively low adult level, when the newborn had sucked (6 hrs of life) The fifth activity, L alanyl L proline dipeptidase, declined successively in the suckling period and reached the adult level first at about 3 weeks *post partum*

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HISTOCHEMISTRY OF THE CILIARY GANGLION OF THE RAT AND THE EFFECT OF PRE- AND POSTGANGLIONIC NERVE DIVISION

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PREFACE

The theme of the present study was proposed in 1964 by Professor Olavi Eranko M D Ph D Head of the Department of Anatomy University of Helsinki This work is part of a research project on the histochemistry of the nervous tissue directed by him

It is a pleasure to express my deep gratitude to Professor Eranko for his supervision encouragement and expert criticism during all stages of the work

I am indebted to Professor Seppo Talanti D V M M L Head of the Department of Anatomy College of Veterinary Medicine Helsinki for his constructive criticism of the manuscript

I wish to express my sincere thanks to Dr Aulikki Kokko M D for many valuable discussions and for her guidance under which the present work was done I am grateful to Dr Martti Ikonen M.D for revision of a part of the manuscript

I owe special thanks to my colleagues in the Department of Anatomy for their pleasant co operation and helpful attitude during this work

My thanks are furthermore due to Miss Paula Alahaivala and Mrs Pirkko Alanko for their excellent technical assistance and to Miss Helmi Palomaki for help in obtaining the literature

Helsinki August 1966

Kauko T Huikuri

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INTRODUCTION

Adamuk (1870) was the first to point out that the ciliary ganglion of mammals was a special type of ganglion, neither sympathetic nor sensory Since then however the nature of this ganglion has been a very controversial subject (Schwalbe 1879 Retzius 1894)

The ciliary ganglion cells have been divided into eight categories (Pines 1927) a classification which is of histological interest but difficult to interpret functionally The majority of the neurones of the mammalian ciliary ganglion are multipolar although some bipolar nerve cells can also be found The neurones which are all of nearly the same size are surrounded by a mantle of satellite cells under which most of the numerous synapses are located The dendritic processes are short forming a plexus almost exclusively under the satellite cell capsule The axon is usually long (Retzius 1894 Slavich 1932)

Enzyme histochemistry supported by modern microbiochemistry has thrown light on the character and function of the ciliary ganglion cells Investigations made by these methods have established that the mammalian ciliary ganglion cells are purely cholinergic that is to say that acetylcholine is the transmitter substance in the synapses of this ganglion (Koelle 1955 Giacobini 1959 Koelle and Koelle 1959 Taxi 1961) On the other hand a few pharmacological studies have indicated that some kind of adrenergic synaptic transmission takes place in this ganglion as well (Tum Suden *et al* 1951 1952 Perry and Talesnik 1953 Perry 1957)

However relatively little work has been done towards elucidating the enzyme histochemistry of the nervous system especially of its parasympathetic part Except in the case of the cholinesterases extremely few investigations concerning the enzymes in the parasympathetic nervous tissue have been made There are still fewer studies concerning the effect of experimental procedures e g nerve division on the behaviour of enzyme activities A systematic investigation of hydrolysing and oxidative enzymes is totally lacking A previous study made in this laboratory on the superior cervical ganglion of the rat (Harkonen 1964) has proved the value of such an approach

The aim of the present study has been in fact to elucidate the histochemistry of enzymes such as carboxylic esterases phosphatases and oxidative enzymes in the ciliary ganglion of the rat under normal conditions and at varying periods after pre and postganglionic denervation or after extirpation of the superior cervical ganglion Catecholamines have also been studied in the normal ciliary ganglion of the rat

GENERAL MATERIAL AND METHODS

Normal Material

The normal material comprised about 200 male and female rats of the Sprague-Dawley strain Their ages ranged from 3 to 12 months The animals were killed by decapitation under light ether anaesthesia Both ciliary ganglia were immediately removed by the intraorbital route as follows After decapitation the head of the rat was skinned The zygomatic arches were cut through and the lateral part of the Harderian gland excised The ganglia were then removed with a small portion of the oculomotor nerve The whole procedure of removing both ganglia took about 5 minutes

For histochemical studies formalin fixed fresh-frozen and freeze-dried ganglia were used Formalin fixation was carried out at 4 C with formol-calcium (Eranko 1959) for 6 hours Thereafter the ganglia were embedded in 10 % gelatin replaced in formol calcium and left overnight After fixation the specimens were cut with a freezing microtome at 8μ These sections were stained free floating For the fresh and postfixed section the newly resected ganglia were mounted in 10 % gelatin on a steel tissue holder and frozen in liquid nitrogen The specimens were sectioned with a cryostat microtome at 8μ These sections were mounted on clean coverslips and dried at room temperature for 10 minutes The fresh sections were then preincubated or incubated Some of the cryostat sections were also postfixed in formol calcium or in acetone in a refrigerator for 15 minutes The freeze drying procedure is described in connexion with the catecholamines (Chapter IV)

Experimental Procedures

Denervation was performed under ether anaesthesia with the aid of a dissecting microscope The left ciliary ganglion was approached by an extracranial route A small skin incision was made perpendicular to the zygomatic arch and a small part of this was resected The lateral part of the Harderian gland was removed or partly drawn out from the orbit The ganglion was denervated in two ways (1) Preganglionically by dissecting the oculomotor nerve about 1 2 mm centrally from the ganglion (decentralization) and (2) postganglionically by cutting all postganglionic nerve fibres (axotomy) The criterion of the success of the operation was maximal dilatation of the ipsilateral pupil Decrease in size of the pupil was used as a criterion of functional recovery

The effect of removal of the left superior cervical ganglion (sympathectomy) on the ciliary ganglion was also studied Therefore a longitudinal incision was made in the neck of the rat and the ganglion was approached bluntly and excised As a criterion of the success of the operation Horner's syndrome in the ipsilateral part of the head was used

In every experiment the contralateral unoperated ganglion was used as a control It was always like the ciliary ganglion of an untreated animal

The postoperative recovery period varied from 1 to 90 days The number of rats killed at different times after the operation is seen in Table I

TABLE I

Ciliary ganglia studied at different times after three types of nerve division

Days after nerve division	Number of rats		
	Decentralization	Axotomy	Sympathectomy
1	6	11	
3	6	12	
5	12	12	10
10	12	14	10
15	12	15	
30	13	15	10
60	13	15	
90	15	16	

I CARBOXYLIC FSTERASES

EARLIER INVLSTIGATIONS

NORMAL NERVOUS TISSUE

Acetylcholinesterase

Acetylcholinesterase (AChE) or true or specific cholinesterase which is known to play an important role in the transmission of nervous impulses is the most widely studied enzyme belonging to the group of carboxylic esterases According to Koelle s hypothesis (1962) acetylcholine (ACh) is the original transmitter substance in cholinergic and noncholinergic neurones This view is supported by studies revealing the presence of AChE which hydrolyses ACh in all nervous tissues (Nachmansohn 1959)

Ciliary Ganglion Intense AChE activity has been reported in the ciliary ganglion of many species The positive AChE reaction obtained when acetylthiocholine is used as substrate in combination with various specific inhibitors has been described in the cytoplasm of the ciliary ganglion cells of the cat (Koelle 1950 1951 1955 Fukuda and Koelle 1959 Koelle and Koelle 1959 McIsaac and Koelle 1959 Cauna *et al* 1961 Taxi 1961 Fredricsson and Sjoqvist 1962) the rabbit the rhesus monkey (Koelle 1955) the hen (Szentagothai *et al* 1955) the chicken (Taxi 1961) the dog the horse and man (Okinaka *et al* 1963)

Two morphologically and enzymatically distinct types of neurones were reported in the ciliary ganglion of the cat (Whitteridge 1937 Koelle 1950) However Koelle s later (1951 1955) investigations and those made by other authors (Fukuda and Koelle 1959 Koelle and Koelle 1959 McIsaac and Koelle 1959 Cauna *et al* 1961 Taxi 1961 Fredricsson and Sjoqvist 1962 Okinaka *et al* 1963) have established that all the ganglion cells in the mammals studied exhibit an intense AChE activity The high activity was uniformly distributed in the ganglion cell cytoplasm and in the cell processes which seemed to be mainly subcapsular dendrites enveloping the cell body In birds in which the ciliary ganglion cells are unipolar (Lenhossék 1911) the cytoplasmic AChE reactivity was moderate in all neurones but the perineuronal activity was exclusively concentrated in one end of the ganglion cell

The high synaptic AChE activity in the ciliary ganglion has been emphasized both in mammals (Koelle 1951 Fukuda and Koelle 1959 Koelle and Koelle 1959 Cauna *et al* 1961 Taxi 1961 Fredricsson and Sjoqvist 1962) and in birds (Szentagothai *et al* 1955 Szentagothai 1957 Taxi 1961) In mammals the activity is distributed in both the pre and postganglionic part of the synapse although the greater part is supposed to be localized postsynaptically (Koelle 1951) In birds the activity is mainly situated

in the calyciform (De Lorenzo 1960 Takahashi and Hama 1965 1965 a) presynaptic part of the synapse although Szentagothai *et al* (1955) found some activity postsynaptically as well

Some nerve fibres also show the AChE activity in the ciliary ganglion (Koelle 1951 1955 Koelle and Koelle 1959 Taxi 1961 Okinaka *et al* 1963) Most of the active fibres are postganglionic while the preganglionic fibres stain very faintly (Koelle 1951 Gerebtzoff 1959 Taxi 1961) although Okinaka *et al* (1963) have demonstrated a very high AChE activity in the oculomotor nerve there being no difference between the autonomic and somatic fibres The AChE positive fibres are less numerous in the ciliary ganglion than in other ganglia (Koelle 1951) However even after prolonged incubation Cauna *et al* (1961) were not able to demonstrate any AChE positive nerve fibres in the ciliary ganglion of the cat

Biochemical studies using a microgasometric technique demonstrated a high AChE activity in all the isolated ciliary ganglion cells of the rat only the anterior horn cells of the spinal cord showed a slightly higher activity The AChE concentration varied very little between the individual cells in contrast with the sympathetic and sensory neurones in which great variation in the activity of this enzyme existed between different cells (Giacobini 1959 1959 a 1960)

Other Nervous Tissues In the sympathetic ganglion cells the AChE activity varies considerably between individual neurones as demonstrated histo- and biochemically A minority of the nerve cells possess a high enzyme activity the rest of the ganglion cells are moderately or lightly stained (Koelle 1951 1955 Giacobini 1956 1957 Snell 1958 Holmstedt and Sjoqvist 1959 Koelle and Koelle 1959 Cauna *et al* 1961 Taxi 1961 Harkonen 1964) This is also the case with sensory nerve cells However some sensory neurones are devoid of any AChE activity (Koelle 1951 1955 Giacobini 1959 a Cauna and Naik 1963 Esila 1963 Tewari and Bourne 1963 Kokko 1965) while motor nerve cells usually exhibit a high AChE activity (Nachmansohn and Hoff 1944 Koelle 1951 1955 Giacobini and Holmstedt 1958 Schwarzacher 1958 Giacobini 1960 Soderholm 1965) Giacobini (1956) has studied histochemically isolated sympathetic and spinal ganglion cells and motor neurones of the rat and noticed the same distribution of AChE activity as described above

A high AChE activity has been demonstrated in the preganglionic axons and their terminals in the sympathetic ganglia (Koelle 1951 1955 Koelle and Koelle 1959 Cauna *et al* 1961 Taxi 1961 Harkonen 1964) while in motor neurones the enzyme activity is high in the pre- and postganglionic axons and synaptic structures (Schwarzacher 1958 Soderholm 1965)

Intracellular Localization The distribution of histochemically demonstrable cytoplasmic AChE activity corresponds to that of the Nissl substance (Fukuda 1959 Fukuda and Koelle 1959) which in turn probably represents the granular endoplasmic reticulum (Palay and Palade 1955) where AChE may be synthesized and thereafter transported to the surface of the cell body and to its processes (Fukuda and Koelle 1959) Biochemical studies based on differential centrifugation have shown this view to be correct AChE being mainly associated with the membranous structures of the microsomal fraction (Nathan and Aprison 1955 Aldridge and Johnson 1959 Hanzon and Toschi 1959 Holmstedt and Toschi 1959 Toschi 1959) Later investigations into the subcellular distribution of AChE ACh and choline acetylase (ChA) have demonstrated that AChE is localized mainly in the membranous part of the nerve endings ACh in

the synaptic vesicles and ChA partly in the synaptic vesicles partly in the free cytoplasm (Whittaker 1959 Gray and Whittaker 1960 De Robertis *et al* 1961 1962 1963 Rodriguez De Lores Arnaiz 1964 Whittaker *et al* 1964 Whittaker and Sheridan 1965) In electron microscopic studies the AChE activity has been observed to be localized in the endoplasmic reticulum in the pre and postsynaptic membranes (Torack and Barrnett 1962 Lewis and Shute 1964 1965 Lewis *et al* 1965 Koelle and Foroglou Kerameos 1965 Shute and Lewis 1966) and in the axonal membrane (Lewis and Shute 1965 a Shute and Lewis 1966)

Non specific Cholinesterase

Non specific cholinesterase (ns ChE) in the ciliary ganglion has been reported to be localized almost exlusively to the satellite cells the Schwann cells and the nerve fibres (Koelle 1951 1955 Cauna *et al* 1961 Taxi 1961) The animals studied were the cat the rabbit the monkey and the hen The ganglion cell cytoplasm was totally devoid of histochemically demonstrable activity These results are supported by biochemical studies on rat ciliary ganglion cells (Giacobini 1959 1960) Csillik and Savay (1954) demonstrated histochemically an intense ns ChE activity in every ciliary ganglion cell of the rat while glial cells and nerve fibres were less strongly stained However the method used by them probably revealed the non specific esterase activity as well

A similar distribution of ns ChE activity has been seen in the sympathetic and sensory ganglion cells of birds and mammals excluding the rat (Koelle 1951 1955 Giacobini 1956 1960 Holmstedt 1957 Cauna *et al* 1961 Taxi 1961 Cauna and Naik 1963) By contrast some sympathetic and sensory neurones of the rat exhibit a moderate ns ChE activity (Giacobini 1959 Cauna *et al* 1961 Taxi 1961 Harkonen 1964 Kokko 1965) while the motor neurones show no ns ChE activity at all (Koelle 1951 1955 Schwarzacher 1958 Giacobini 1959 1960 Soderholm 1965)

Intracellularly ns ChE is mainly distributed in the nuclear microsomal and supernatant fractions (Aldridge and Johnson 1959) Ultrastructurally ns ChE is mainly localized in the endoplasmic reticulum in the hypoglossal neurones of the rat (Lewis and Shute 1965 a)

Non specific Esterases

The division of the non specific esterases (ns Es) into E 600-sensitive non specific esterase (E-s ns E) and E 600 resistant non specific esterase (E-r ns E) according to whether or not they are inhibited by diethyl p-nitrophenyl phosphate (E 600) has seldom used in the previous investigations which makes their interpretation difficult

Savay *et al* (1953) using β naphthyl acetate as substrate and acetone fixed paraffin sections described an intense ns E activity in nearly all the ciliary ganglion cells and cell processes of the cat The glial cells and nerve fibre plexuses around the neurones were moderately positive while the postganglionic nerve fibres were negative

The ns E activity varies between individual nerve cells in the sympathetic spinal and other ganglia of the rabbit dog and cat (Savay *et al* 1953) In the rat the E-s ns E activity is mainly localized in the cytoplasm of the sympathetic (Harkonen 1964) spinal

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(Thomas 1963 Kokko 1965) and other ganglion cells (Fisher and Sutherland 1965 1965 a) and the activity varies moderately between individual neurones while in the motor nerve cells this variation is less distinct (Soderholm 1965) The activity of E r ns E is much weaker than that of E s ns E and it is mainly localized in the cytoplasmic granules in the nerve cells (Harkonen 1964 Fisher and Sutherland 1965 a Kokko 1965 Soderholm 1965)

Biochemical studies have demonstrated that the mitochondrial and microsomal fractions possess the highest ns E activity in the brain tissue (Aldridge and Johnson 1959 Sellinger and De Balbian Verster 1962) Electron microscopic studies have revealed that E s ns E is localized in the nuclear membrane endoplasmic reticulum and Golgi apparatus its distribution resembling that of ns ChE while E r ns E is mostly situated in dense bodies in the neurones and neuroglial cells of the rat brain tissue (Torack and Barrnett 1962)

EFFECT OF DENERVATION

Preganglionic Denervation

Acetylcholinesterase Division of the preganglionic nerve trunk of the ciliary ganglion in the hen and the chick was reported to result in the disappearance of AChE activity in the preganglionic synaptic structures the postganglionic synaptic activity remaining intact (Szentágothai *et al* 1955 Taxi 1961) The change in enzyme activity was seen after 2 days and reached a maximum within a week after the operation In the ciliary ganglion of the cat a considerable decrease in the number of active intraganglionic nerve fibres was observed after decentralization In addition to this the enzyme activity at the periphery of the neurones was reported by Koelle and Koelle (1959) to be much decreased while Taxi (1961) did not find any marked change in the reactivity of this synaptic zone However some formerly active ganglion cells lost much of their reactivity which may have been due to operational interference with the post ganglionic fibres (Taxi 1961)

Decentralization also brings about the disappearance of AChE activity from the preganglionic nerve fibres and their terminals in the sympathetic ganglia of the rat (Brown 1958 Taxi 1961 Harkonen 1964) and the cat (Koelle 1955 Snell 1958 Koelle and Koelle 1959 Fredricsson and Sjoqvist 1962 Holmstedt *et al* 1963)

Biochemical studies have confirmed that after decentralization a definite decrease in the enzyme activity occurs in the sympathetic ganglia of the rat (Dhar 1958 Harkonen 1964) and the cat (von Brucke 1937 Sawyer and Hollinshead 1945 Holmstedt *et al* 1963 Gromadzki and Koelle 1965)

Non specific Cholinesterase Taxi (1961) found no change in the ns ChE activity in the ciliary ganglion of the cat after decentralization and the result was the same with the superior cervical ganglion of the rat and the cat (Taxi 1961 Fredricsson and Sjoqvist 1962) Other authors on the contrary have demonstrated a marked decrease in the ns ChE activity in the sympathetic ganglia of the rat and the cat as studied histo- and biochemically (Dhar 1958 Snell 1958 Harkonen 1964)

Non specific Esterases Neither E s ns E nor E r ns E showed any histo- or biochemically demonstrable changes in activity in the superior cervical ganglion of the rat after preganglionic nerve division (Harkonen 1964) No report is available concerning the ciliary ganglion and ns Es after this type of operation

Postganglionic Denervation

Acetylcholinesterase Szentágothai *et al* (1955) did not notice any change in the AChE activity in the ciliary ganglion of the hen after axotomy while Taxi (1961) described a distinct decrease of the enzyme activity in the ciliary ganglion cells of the cat and the chick In the former species nearly all the AChE activity disappeared from the ganglion cell cytoplasm in 10 days while the perineuronal synaptic activity was affected much less than after decentralization

After axotomy a great diminution in histochemically demonstrable AChE activity in the neuronal cytoplasm synapses and postganglionic nerve fibres of the sympathetic ganglia has been observed in the rat (Brown 1958 Taxi 1961 Harkonen 1964 Eranko and Harkonen 1965) and the cat (Fredricsson and Sjoqvist 1962 Gromadzki and Koelle 1965) Similar changes have been found in the motor neurones of the rat (Schwarzacher 1958 Soderholm 1965) and the toad (Chacko and Cerf 1960) In addition Harkonen (1964) and Soderholm (1965) have reported a loss of AChE from the preganglionic nerve fibres and synaptic terminals after axotomy

These findings have been confirmed by biochemical studies concerning the AChE content of the axotomized sympathetic ganglia of the rat (Dhar 1958 Harkonen 1964) and the cat (Sawyer and Hollinshead 1945 Brown *et al* 1952 McLennan 1954 Gromadzki and Koelle 1965) Lewis and Shute (1965 a) have reported a great decrease of AChE activity in the hypoglossal neurones of the rat after axotomy as studied with the electron microscope Many nerve fibres proximal to the cut exhibited foldings of the axonal membrane with enhanced enzyme activity while in the distal segment there was complete loss of enzyme activity

Non specific Cholinesterase A loss of this enzyme from the neuronal cytoplasm and a marked decrease of activity in the glia were demonstrated by Harkonen (1964) in the axotomized superior cervical ganglion of the rat whereas Taxi (1961) and Fredricsson and Sjoqvist (1962) failed to observe any such change after axotomy in the enzyme activity of the sympathetic ganglia of the rat and the cat respectively In the central nervous system in which the activity is restricted almost exclusively to the capillar es ns ChE has not been observed to undergo any change after axotomy (Schwarzacher 1958 Soderholm 1965)

Biochemical determinations have verified that a definite decrease in ns ChE activity takes place in the sympathetic ganglia of the rat (Dhar 1958 Harkonen 1964) and the cat (Sawyer and Hollinshead 1945) after axotomy In the latter case almost total disappearance of the activity of the enzyme was observed Electron microscopically it was seen that almost all the ns ChE activity disappeared from the cytoplasm of the neurones of the hypoglossal nuclei of the rat after axotomy (Lewis and Shute 1965 a)

Non specific Esterases The E-s ns E activity in the sympathetic ganglion cells of the rat diminished after axotomy but there was no such change in the E r ns E activity (Harkonen 1964) In the anterior horn cells of the spinal cord of the rat both these

enzymes showed a markedly decreased activity after postganglionic denervation (Soderholm 1965) while in the spinal cord of the guinea pig only a slight decrease of ns E activity was observed with naphthol AS-acetate as substrate after sciatic neurotomy (Kumamoto and Bourne 1963) After an initial increase in the nodose ganglion and dorsal vagal nucleus of the monkey following cervical vagotomy a fall in the organophosphate resistant indoxyl esterase activity to below the normal level was described by Fisher and Sutherland (1965 a)

Biochemically diminished activity of both E s ns E and E r ns E was demonstrated in the sympathetic ganglion of the rat after axotomy although the latter enzyme had lost only about 14 % of its activity (Harkonen 1964)

Sympathectomy

Csillik and Koelle (1965) described a slight reduction (15—20 %) in the number of AChE positive nerve fibres in the rat iris after extirpation of the ipsilateral superior cervical ganglion while Ehinger and Falck (1965) did not observe any change in the number or staining intensity of the AChE positive fibres in the rat iris after removal of the cervical sympathetic chain There is no report available about the effect of these operations on the enzyme activity of the ciliary ganglion

METHODS

Cholinesterases

Gomori s (1952) modification of Koelle s (1951) method was used for the demonstration of cholinesterases Sections were preincubated in the stock solution with or without inhibitor for 30 minutes at 37°C and thereafter incubated for 2—6 hours in the same medium to which the substrate acetyl or butyrylthiocholine iodide was added

To differentiate between acetylcholinesterase and non specific cholinesterase (Augustinson and Nachmansohn 1949) the following inhibitors were used 1 5 bis-(4 allyl dimethylammoniumphenyl) pentan-3 one-diiodide (284 C 51) in a concentration of 10 M to inhibit AChE selectively (Austin and Berry 1953 Bayliss and Todrick 1956) and tetra isopropylpyrophosphoramide (*iso*-OMPA) in a concentration of 10 *M to inhibit ns ChE selectively (Aldridge 1953 Austin and Berry 1953 Bayliss and Todrick 1956 Diegenbach 1965) The concentrations of these inhibitors were checked before the routine procedures

Control sections were incubated without the substrate or by using both 10 *M *iso*-OMPA and 10 M 284 C 51 together or 10 ³M physostigmine (eserine) alone to inhibit ChEs (Augustinson 1948 Gomori 1952 Pearse 1960) These controls were always totally negative

Non specific Esterases

Non specific esterases can be divided into two groups as in previous studies made in this laboratory (1) E 600-sensitive non specific esterase and (2) E 600-resistant non specific esterase according to their behaviour towards E 600 as inhibitor (Aldridge 1953 Eranko *et al* 1962 1964 Harkonen 1964 Kokko 1965 Soderholm 1965) E-s ns E which is eserine resistant is selectively demonstrated in fresh sections and E-r ns E in fixed sections (Eranko *et al* 1964 Harkonen 1964 Kokko 1965 Soderholm 1965)

The sections were preincubated with or without any inhibitor in a buffer solution at the same pH as that of the incubation medium for 30 minutes before incubation

In addition to the above mentioned inhibitors eserine in a concentration of 10 ⁵M was used to inhibit both ChEs (Richter and Croft 1942 Augustinson 1948 Summer and Myrback 1950 Aldridge 1953 a) E 600 (Paroxon Mintacol) 10 M was used to differentiate between E s ns E and E-r ns E (Aldridge and Davison 1952 Aldridge 1954 Hobbiger 1957 Eranko *et al* 1964) AChE was inhibited with 10 ⁵M 284 C 51 and ns ChE with 10 ⁴ iso-OMPA

The substrates used were α naphthyl acetate and butyrate 4-chloro-5 bromoindoxyl acetate and naphthol AS D acetate

α Naphthyl Acetate and Butyrate The method described by Nachlas and Seligman (1949) was used as later modified by Pearse (1960) and Eranko *et al* (1962) The incubation time was 3 5 minutes with α naphthyl acetate and 25 minutes with α naphthyl butyrate Blue RR Salt was used as coupling agent

4-Chloro-5 bromoindoxyl Acetate The method used was that of Holt (1952) and Holt and Withers (1952) The incubation time was 75 minutes for fresh and postfixed sections and 45 minutes for fixed sections

Naphthol AS D Acetate Planteydt s (1961) modification of Gossner s method (1958) was employed except that Blue RR Salt was used as coupler The incubation time was 2 hours for fresh and postfixed sections and 35 minutes for fixed sections

The control for all these reactions were made by incubating the sections without either the substrate or the coupling agent These controls were always negative

RESULTS

NORMAL GANGLION

In the presentation of the results the carboxylic esterases are classified according to the principle adopted in this laboratory (Eranko *et al* 1964 Harkonen 1964 Kokko 1965 Soderholm 1965) The esterases are divided into two main groups ChEs and ns Es ChEs are subdivided into AChE and ns ChE using *iso* OMPA and 284 C 51 as specific inhibitors Ns Es are grouped into E s ns E and E r ns E using E 600 as differentiating inhibitor

The most reliable methods for demonstrating the four groups of carboxylic esterases are presented in the following scheme

Enzyme	Type of section	Substrate	Inhibitor	
AChE	Fresh postfixed fixed	Acetylthiocholine iodide 6 9 x 10 ^{3}M	*iso*-OMPA	10 ^{6}M
Ns ChE	Fresh postfixed fixed	Butyrylthiocholine iodide 6 3 x 10 M	284 C 51	10 ^{5}M
E s ns E	Fresh	α Naphthyl acetate 5 4 x 10 ^{3}M	Eserine	10 ^{5}M
E r ns E	Fixed	α Naphthyl acetate 5 4 x 10 ^{3}M	E 600	10 ^{5}M

In the present study the results of estimations of esterase activities in the normal ganglion using different substrates and substrate—inhibitor combinations are summarized in Tables II IV The intensities of the reactions are only studied visually and in the interpretation of the results the following signs are used — ± + ++ +++ which can be read as negative very weak or just visible weak moderate and intense

When naphthol AS D acetate was used as substrate the reaction product was coarsely granular throughout and in every type of section so that it was very difficult to determine the exact site of the activity Therefore little attention is paid to the results obtained with this substrate in the following text

No differences between the sexes were observed in the present study

TABLE II *The intensities of the esterase reactions obtained with different substrates and substrate inhibitor combinations in fresh sections*

Substrate and Inhibitor	Ganglion Cell Cytoplasm	Synapses	Satellite Cells	Nerve Fibres
Acetylthiocholine	— to +	+ +	+ + +	+ + +
iso OMPA 10^{-6}M	— to +	+ +	—	some + +
284 C 51 10^{-5}M	— to +	—	+ + +	+ + +
Eserine 10^{-5}M	—	—	—	—
E 600 10^{-5}M	—	—	—	—
Butyrylthiocholine	some + +	—	+ + +	+ + +
iso OMPA 10^{-6}M	—	—	—	—
284 C 51 10^{-5}M	some + +	—	+ + +	+ + +
Eserine 10^{-5}M	—	—	—	—
E 600 10^{-5}M	—	—	—	—
α Naphthyl acetate	+ + +	+ + +	+ + +	+ + +
iso OMPA 10^{-6}M	+ + +	—	—	+
284 C 51 10^{-5}M	+ + +	—	+ + +	+ + +
Eserine 10^{-5}M	+ + +	—	+	+
E 600 10^{-5}M	—	—	—	—
α Naphthyl butyrate	+ +	—	+ +	+ +
iso OMPA 10^{-6}M	+ +	—	—	+
284 C 51 10^{-5}M	+ +	—	+ +	+ +
Eserine 10^{-5}M	+ +	—	±	±
E 600 10^{-5}M	—	—	—	—
4 Chloro 5 bromo-indoxyl acetate	+ + +	—	+ +	+ +
iso OMPA 10^{-6}M	+ + +	—	—	+ to + +
284 C 51 10^{-5}M	+ + +	—	+ +	+ +
Eserine 10^{-5}M	+	—	—	—
E 600 10^{-5}M	—	—	—	—
Naphthol AS D acetate	+	—	+	+
iso OMPA 10^{-6}M	+	—	+	+
284 C 51 10^{-5}M	+	—	+	+
Eserine 10^{-5}M	+	—	+	+
E 600 10^{-5}M	+	—	—	—

RESULTS

NORMAL GANGLION

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The most reliable methods for demonstrating the four groups of carboxylic esterases are presented in the following scheme

Enzyme	Type of section	Substrate	Inhibitor	
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Ns ChE	Fresh postfixed fixed	Butyrylthiocholine iodide 6 3 x 10 ^{3}M	284 C 51	10 ^{5}M
E-s ns E	Fresh	α Naphthyl acetate 5 4 x 10 ^{3}M	Eserine	10 ^{5}M
E-r ns E	Fixed	α Naphthyl acetate 5 4 x 10 M	E 600	10 ^{5}M

In the present study the results of estimations of esterase activities in the normal ganglion using different substrates and substrate—inhibitor combinations are summarized in Tables II IV The intensities of the reactions are only studied visually and in the interpretation of the results the following signs are used — ± + ++ +++ which can be read as negative very weak or just visible weak moderate and intense

When naphthol AS D acetate was used as substrate the reaction product was coarsely granular throughout and in every type of section so that it was very difficult to determine the exact site of the activity Therefore little attention is paid to the results obtained with this substrate in the following text

No differences between the sexes were observed in the present study

TABLE II *The intensities of the esterase reactions obtained with different substrates and substrate-inhibitor combinations in fresh sections*

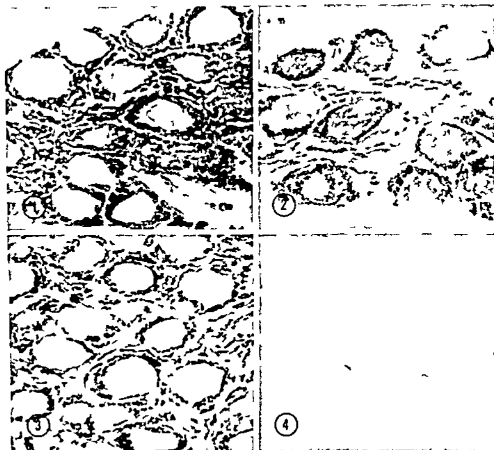
Substrate and Inhibitor		Ganglion Cell Cytoplasm	Synapses	Satellite Cells	Nerve Fibres
Acetylthiocholine		— to +	++	+++	+++
iso OMPA	10^{-6}M	— to +	++	—	some ++
284 C 51	10^{-5}M	— to +	—	+++	+++
Eserine	10^{-5}M	—	—	—	—
E 600	10^{-5}M	—	—	—	—
Butyrylthiocholine		some ++	—	+++	+++
iso OMPA	10^{-6}M	—	—	—	—
284 C 51	10^{-5}M	some ++	—	+++	+++
Eserine	10^{-5}M	—	—	—	—
E 600	10^{-5}M	—	—	—	—
α Naphthyl acetate		+++	+++	+++	+++
iso OMPA	10^{-6}M	+++	—	—	+
284 C 51	10^{-5}M	+++	—	+++	+++
Eserine	10^{-5}M	+++	—	+	+
E 600	10^{-5}M	—	—	—	—
α Naphthyl butyrate		++	—	++	++
iso OMPA	10^{-6}M	++	—	—	+
284 C 51	10^{-5}M	++	—	++	++
Eserine	10^{-3}M	++	—	±	±
E 600	10^{-5}M	—	—	—	—
4 Chloro-5 bromo indoxyl acetate		+++	—	++	++
iso OMPA	10^{-4}M	+++	—	—	+ to ++
284 C 51	10^{-5}M	+++	—	++	++
Eserine	10^{-5}M	+	—	—	—
E 600	10^{-3}M	—	—	—	—
Naphthol AS D acetate		+	—	+	+
iso OMPA	10^{-6}M	+	—	+	+
284 C 51	10^{-5}M	+	—	+	+
Eserine	10^{-3}M	+	—	+	+
E 600	10^{-5}M	+	—	—	—

TABLE III *The intensities of the esterase reactions obtained with different substrates and substrate inhibitor combinations in postfixed sections*

Substrate and Inhibitor		Ganglion Cell Cytoplasm	Synapses	Satellite Cells	Nerve Fibres
Acetylthiocholine		— to ++	+++	+++	+++
iso OMPA	10^{-6}M	— to ++	+++	—	some ++
284 C 51	10^{-5}M	— to +	—	+++	+++
Eserine	10^{-5}M	—	—	—	—
E 600	10^{-5}M	—	—	—	—
Butyrylthiocholine		some ++	—	+++	+++
iso OMPA	10^{-6}M	—	—	—	—
284 C 51	10^{-5}M	some ++	—	+++	+++
Eserine	10^{-5}M	—	—	—	—
E 600	10^{-5}M	—	—	—	—
α Naphthyl acetate		++	—	++	++
iso OMPA	10^{-6}M	++	—	—	—
284 C 51	10^{-5}M	++	—	++	++
Eserine	10^{-5}M	+	—	±	±
E 600	10^{-5}M	—	—	—	—
α Naphthyl butyrate		++	—	++	++
iso OMPA	10^{-6}M	++	—	+	some +
284 C 51	10^{-5}M	++	—	++	++
Eserine	10^{-5}M	++	—	±	±
E 600	10^{-5}M	—	—	—	—
4 Chloro-5 bromo-indoxyl acetate		+	—	++	++
iso OMPA	10^{-6}M	+	—	—	some +
284 C 51	10^{-5}M	+	—	++	++
Eserine	10^{-5}M	+	—	—	+
E 600	10^{-5}M	—	—	—	—
Naphthol AS-D acetate		+	—	+	+
iso OMPA	10 M	+	—	+	+
284 C 51	10 M	+	—	+	+
Eserine	10 M	+	—	+	+
E 600	10 M	+	—	+	+

TABLE IV *The intensities of the esterase reactions obtained with different substrates and substrate inhibitor combinations in fixed sections*

Substrate and Inhibitor	Ganglion Cell Cytoplasm	Synapses	Satellite Cells	Nerve Fibres
Acetylthiocholine	+ to ++	+++	+++	+++
iso OMPA 10^{-6}M	+ to ++	+++	—	some ++
284 C 51 10^{-5}M	— to +	—	+++	+++
Eserine 10^{-5}M	—	—	—	—
E 600 10^{-5}M	—	—	—	—
Butyrylthiocholine	some ++	—	++	+++
iso OMPA 10^{-6}M	—	—	—	—
284 C 51 10^{-5}M	some ++	—	+++	+++
Eserine 10^{-5}M	—	—	—	—
E 600 10^{-5}M	—	—	—	—
α Naphthyl acetate	++	—	++	+++
iso OMPA 10^{-6}M	++	—	—	some +
284 C 51 10^{-5}M	++	—	+++	+++
Eserine 10^{-5}M	++	—	±	±
E 600 10^{-5}M	+	—	—	—
α Naphthyl butyrate	++	—	++	++
iso OMPA 10^{-6}M	++	—	++	+
284 C 51 10^{-5}M	++	—	++	++
Eserine 10^{-5}M	++	—	+	+
E 600 10^{-5}M	+	—	—	—
4 Chloro 5 bromo indoxyl acetate	++	—	++	++
iso OMPA 10^{-6}M	++	—	—	some +
284 C 51 10^{-5}M	++	—	++	++
Eserine 10^{-5}M	++	—	±	±
E 600 10^{-5}M	+	—	—	—
Naphthol AS D acetate	++	—	+	+
iso OMPA 10^{-6}M	++	—	+	+
284 C 51 10^{-5}M	++	—	+	+
Eserine 10^{-5}M	++	—	+	+
E 600 10^{-5}M	++	—	+	+



Figs 1—4 Esterase reactions obtained with acetylthiocholine Incubation time 4 hours Postfixed sections ×420

Fig 1 No inhibitor
Fig 2 *Iso* OMPA 10 M (AChE)
Fig 3 284 C 51 10 M
Fig 4 Eserine 10 ⁵M

Acetylcholinesterase

Effect of Inhibitors In the demonstration of AChE activity in the ciliary ganglion of the rat *iso* OMPA had to be used to inhibit its ChE the activity of which was very intense in the nerve fibres satellite cells and glial cells as can be seen in Fig 1 The concentration of *iso* OMPA generally used was 10 ⁶M If the concentration was 10^{-5}M or higher *iso* OMPA inactivated a considerable part of the AChE activity in the ganglion cell cytoplasm and in the synapses On the other hand if the concentration of *iso* OMPA was

lower than 10^{-6}M the glial tissue exhibited rather strong activity 10^{-5}M 284 C 51 totally inhibited the AChE activity in the synapses and positive nerve fibres and in the majority of the ganglion cells (Fig 3) 10^{-5}M eserine and 10^{-5}M E 600 totally inhibited the reaction obtained with acetylthiocholine (Fig 4)

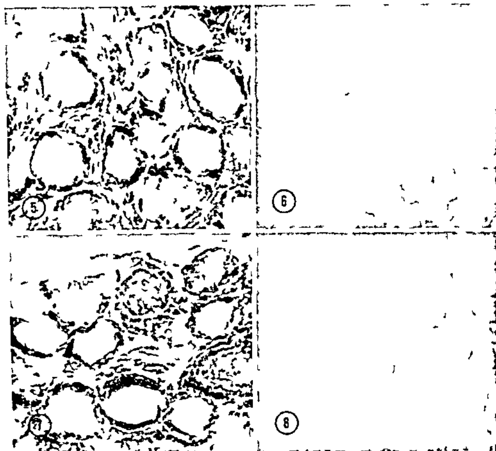
Other Substrates Butyrylthiocholine was not hydrolysed by AChE since 10^{-5}M 284 C 51 had no effect on the reactivity towards this substrate (Figs 5 and 7) and 10^{-6}M *iso* OMPA completely inhibited it (Fig 6) In fresh sections α naphthyl acetate gave a reaction which was partly suppressed by 10^{-5}M 284 C 51 (Table II) The inhibition involved the ganglion cell cytoplasm the synaptic zone, and some nerve fibres (Figs 9 and 11) Since no activity was seen in the synaptic area when 10^{-6}M *iso* OMPA was used as inhibitor (Table II Fig 10) the activity of the synaptic zone revealed by α naphthyl acetate must have been due to ns ChE in the satellite cells In postfixed and fixed section α naphthyl acetate was not hydrolysed by AChE since 10^{-5}M 284 C 51 had no effect on the reaction (Tables III and IV)

Distribution As observed with acetylthiocholine and 10^{-6} *iso* OMPA the most intense activity was present in the synapses which appeared like a ring around the neurones (Tables II III and IV Figs 2 21 and 33) These synaptic zones were not always continuous all the way round the cell body but less intensely stained areas were observed at the cell periphery between the synaptic clumps An almost equally strong staining as in the synapses was seen in the cytoplasm of some ganglion cells but there were only a few of these in each ganglion The cytoplasm of other neurones showed a moderate activity in postfixed and fixed sections but only weak to moderate activity in fresh sections The staining was evenly distributed throughout the whole cytoplasm and the reaction product was finely granular In some neurones the nuclear membrane exhibited a more intense activity than the cytoplasm The satellite cells and glial cells were inactive as well as the nuclei and nucleoli but some nerve fibres were intensely reactive In the interstitium between the ganglion cell bodies some strongly positive clumps were observed These were presumed to be synapses of ganglion cells the nucleus and most of the cytoplasm of which were outside the section

A positive reaction was observed in the synapses after incubation for 30 minutes after longer incubation other parts of the ganglion became positive When the incubation time was six hours the distribution of the activity did not change although the intensity of the reaction increased In fresh sections the activity was weaker and the reaction product more diffuse than in postfixed and fixed sections (Tables II III and IV)

Non specific Cholinesterase

Effect of Inhibitors When butyrylthiocholine was used as substrate and 10^{-5}M 284 C 51 as inhibitor the result was similar to that obtained without an inhibitor, establishing that only ns ChE hydrolysed butyrylthiocholine



Figs 5—8 Esterase reactions obtained with butyrylthiocholine Incubation time 3 hours Postfixed sections x 420

Fig 5 No inhibitor
Fig 7 284 C 51 10 ⁵M (ns ChE)
Fig 6 Iso-OMPA 10 ⁶M
Fig 8 E 600 10 ⁵M

(Figs 5 7 27 and 39) 10 ⁶M *iso* OMPA 10^{-5}M eserine and 10 M E 600 inhibited all the activity obtained with this substrate (Figs 6 and 8)

Other Substrates The result with acetylthiocholine as substrate and 10^{-5}M 284 C 51 as inhibitor was similar to that obtained with butyrylthiocholine alone (Tables II III and IV Figs 3 and 5) This reaction was totally inactivated by 10 ⁶M *iso* OMPA α Naphthyl acetate α naphthyl butyrate and 4-chloro 5 bromoindoxyl acetate were also hydrolysed by ns ChE but these reactions were not totally inhibited by 10 ⁶M *iso* OMPA (Tables II III and IV Figs 10 and 16) Neither did 10 ⁵M 284 C 51 or 10^{-4}M eserine totally abolish the activity in the cytoplasm of the ganglion cells (Figs 9 11 15 17 and 20)

Distribution As shown with butyrylthiocholine the distribution of ns ChE activity was similar in all types of sections although in postfixed and fixed sections the picture was sharper than in fresh sections (Tables II III and IV Figs 5 7 27 and 39) Only a few of the ganglion cells (1 5 cells per ganglion) were stained moderately to intensely, the other neurones were totally devoid of activity On the other hand the satellite cells Schwann cells and most of the nerve fibres exhibited intense activity The reaction product was finely granular The nuclei and nucleoli of all these types of cells were inactive

The satellite cells appeared as an intensely stained zone surrounding the ganglion cell bodies like the synaptic ring Therefore it was difficult to distinguish whether the activity of this zone was due to that of the synapses or of the satellite cells or of both of these To solve this problem sections stained separately for AChE or ns ChE were counterstained with haematoxylin From the original sections it was observed that there are not at any rate large amounts of ns ChE capable of hydrolysing butyrylthiocholine present in the synapses The sections counterstained with haematoxylin established this to be true the ns ChE activity around the ganglion cell bodies was confined to the satellite cells

E 600 sensitive Non specific Esterase

Effect of Inhibitors E 600 in a concentration of 10^{-5}M totally inhibited the activity obtained with α naphthyl acatate in fresh and postfixed sections (Tables II III and IV Fig 13) In fixed sections some activity due to E r ns E remained

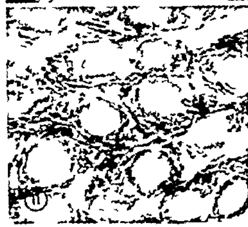
Other Substrates When α naphthyl butyrate and 4-chloro 5 bromoindoxyl acetate were used as substrates and 10^{-5}M eserine as inhibitor the distribution of the activity was similar to that observed with α naphthyl acetate in fresh postfixed and fixed sections although the intensity of the reaction was a little weaker and the reaction product more coarsely granular than with α naphthyl acetate (Tables II III and IV Fig 18) 10^{-5}M E 600 totally inhibited the reaction obtained with these other two substrates in fresh and postfixed sections while in fixed sections the result was closely similar to that obtained with α naphthyl acetate and E 600 (Figs 14 and 19)

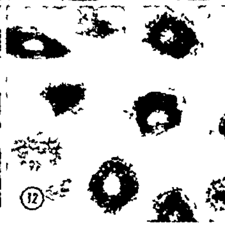
Distribution The enzyme was mainly restricted to the cytoplasm of ganglion cells the majority of these showing intense activity but ome neurones were moderately stained (Figs 12 and 45) The reaction product which was evenly distributed throughout the whole cytoplasm was partly diffuse and partly finely granular and extended some way into the cell processes so that the multipolarity of the ganglion cells could be seen Weak activity was visible in the cytoplasm of the satellite cells and in some nerve fibres between the ganglion cell bodies The latter interstitial activity may be partly due to that of the Schwann cells The nuclei and nucleoli were not reactive

In the postfixed and fixed sections the distribution of activity was similar to that in the fresh sections but the intensity was a little weaker (Tables







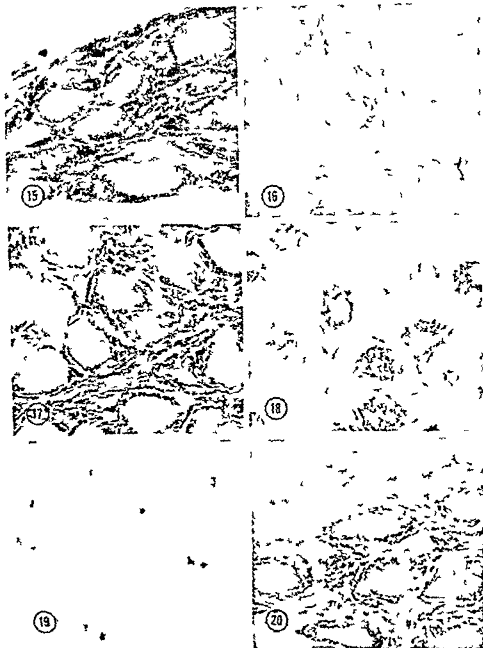


13

14

Figs 9—14 Esterase reactions obtained with α naphthyl acetate and Blue RR Incubat[ion] time 15 min *Figs 9—13* Fresh sections *Fig 14* Fixed section x 420

Fig 9 No inhibitor
Fig 10 *Iso*-OMPA 10 M
Fig 11 284 C 51 10 M
Fig 12 Eserine 10 M (Es ns F)
Fig 13 E 600 10 M
Fig 14 E 600 10 M (Es ns E)



Figs 15—19 Esterase reactions obtained with α-naphthyl butyrate and Blue RR. Incubation time 25 min *Figs 15—18* Postfixed sections *Fig 19* Fixed section ×420 *Fig 20* Esterase reaction obtained with 4-chloro-5 bromoindoxyl acetate Incubation time 75 min Postfixed section ×420

Fig 15 No inhibitor

Fig 16 *Iso*-OMPA 10 M

III and IV) than in the fresh sections In addition to this in fixed sections in the interstitium between the ganglion cell bodies, many small and very intensely stained spots were visible (macrophages fibrocytes capillaries?) which had not been affected by any inhibitor (Figs 14 and 19)

E 600 resistant Non specific Esterase

This enzyme was selectively demonstrated in fixed sections using α naphthyl acetate as substrate and 10^{-5}M E 600 as inhibitor

Other Substrates In fixed sections, α naphthyl butyrate and 4 chloro 5 bromoindoxyl acetate as substrates, together with 10^{-5}M E 600 as inhibitor gave almost the same picture as that obtained with α naphthyl acetate except that the intensity of activity was weaker with the two former than with the latter (Fig 19) In fresh and postfixed sections no reaction at all was obtained (Tables II and III)

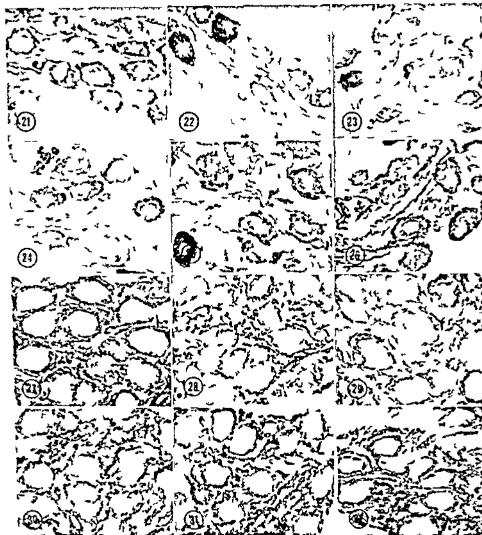
Distribution All the ganglion cells showed some activity which was mainly localized in coarse granules but a weak background activity was also seen The granules were mostly situated perinuclearly as a broad zone but in some neurones the reaction product was concentrated in one end of the ganglion cell (Fig 14) The satellite cells the glial cells and the nerve fibres as well as all nuclei and nucleoli were totally devoid of activity In the interstitium between the ganglion cell bodies several intensely stained small clumps were seen These were homogeneously and heavily stained so that it was impossible to discern the fine structure or determine the nature of these spots

PREGANGLIONIC DENERVATION

Acetylcholinesterase The number of AChE positive synapses around the ganglion cells decreased after the operation This was first seen on the 3rd day after nerve section and the loss reached a maximum by the 5th day (Figs 21 and 22) Thereafter the number of reactive synapses remained subnormal for about a fortnight (Figs 23 and 24) subsequently gradually returning to normal so that after 60 days the synaptic zones were as densely stained as in the control sections (Figs 25 and 26) The partial weakening of the reaction in the synaptic zone may be due to the degeneration of the preganglionic part of the synapse

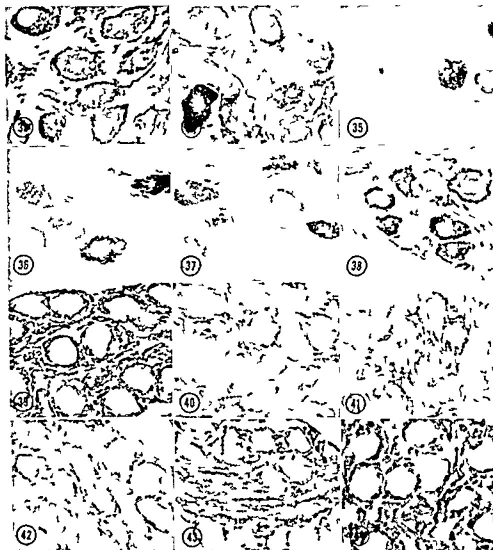
The cytoplasmic AChE activity remained substantially unchanged However this activity seemed to increase during the regeneration period (Figs 25 and 26) The number of active fibres did not change as a result of decentralization

Non specific Cholinesterase The ns ChE activity began to decrease 3 days after section of the preganglionic nerve trunk and maximum loss was reached in 5 days (Figs 27 28 29 and 30) The formerly strongly positive nerve fibres had lost the greater part of their activity although this decrease



The effect of preganglionic denervation on AChE and ns ChE activities Incubation time 4 hours for AChE and 3 hours for ns ChE Postfixed sections x 250

Fig 21 AChE Control ganglion
Fig 22 AChE 5 days after decentralization
Fig 23 AChE 10 days after decentralization
Fig 24 AChE 30 days after decentralization
Fig 25 AChE 60 days after decentralization
Fig 26 AChE 90 days after decentralization
Fig 27 Ns ChE Control ganglion
Fig 28 Ns ChE 5 days after decentralization
Fig 29 Ns ChE 10 days after decentralization
Fig 30 Ns ChE 15 days after decentralization
Fig 31 Ns ChE 30 days after decentralization
Fig 32 Ns ChE 90 days after decentralization



The effect of postganglionic denervation on AChE and ns ChE activities Incubation time 4 hours for AChE and 3 hours for ns ChE Postfixed sections x 250

Fig 33 AChE Control ganglion
Fig 34 AChE 1 day after axotomy
Fig 35 AChE 3 days after axotomy
Fig 36 AChE 15 days after axotomy
Fig 37 AChE 30 days after axotomy
Fig 38 AChE 60 days after axotomy
Fig 39 Ns ChE Control ganglion
Fig 40 Ns ChE 3 days after axotomy
Fig 41 Ns ChE 5 days after axotomy
Fig 42 Ns ChE 10 days after axotomy
Fig 43 Ns ChE 30 days after axotomy
Fig 44 Ns ChE 90 days after axotomy

was not so great as that caused by axotomy The weakening of the fibre activity was due to degeneration of the preganglionic fibres The activity was partly restored by the 30th day (Fig 31) and totally normalized by the 60th day after nerve section (Fig 32) However in some ganglia the number of active fibres was less than in normal ganglia

E 600 sensitive and resistant Non specific Esterases No distinct changes were found in the activity of either of these enzymes in the ciliary ganglion of the rat after preganglionic denervation

POSTGANGLIONIC DENERVATION

Acetylcholinesterase The first changes in the activity of this enzyme were already observable on the first postoperative day The distribution of the AChE reaction in the axotomized ganglion was less sharp than in the control and the cytoplasmic activity of its neurones was clearly weakened (Figs 33 and 34) After three days most of the ganglion cells were totally negative except for a few neurones whose activity was hardly affected (Fig 35) The synaptic reactivity had also diminished within less than a day after the operation and AChE positive synapses were less numerous On the 3rd postoperative day only a few reactive synapses were visible and no AChE positive nerve fibres were seen The staining remained faint during the following weeks (Fig 36) but by the 30th day after axotomy activity was somewhat restored (Fig 37) Two months after the operation the AChE activity in the ganglion cells was at least as strong as in the control ganglion (Fig 38) By this time synapses and nerve fibres containing AChE had also reappeared

During the axon reaction the ganglion cell bodies became oval and the nuclei moved to the periphery of the neurone These phenomena had also normalized 60 days after the operation

Non specific Cholinesterase After 3 days the ns ChE positive nerve fibres had lost their activity (Figs 39 and 40) although some fibres probably preganglionic were still as strongly positive as in the control section No active ganglion cells were seen either The staining remained faint during the following period (Figs 41 and 42) and gradually began to increase having returned somewhat by the 30th day (Fig 43) and the activity was totally normalized 60 days after the operation (Fig 44) The satellite and Schwann cells also lost some of their activity during the axon reaction



The effect of postganglionic denervation on E-s ns E activity Incubation time 3.5 min Fresh sections x 250

Fig 45 E-s ns E Control ganglion
Fig 46 E s ns E 1 day after axotomy
Fig 47 E s ns E 3 days after axotomy
Fig 48 E s ns E 5 days after axotomy
Fig 49 E s ns E 30 days after axotomy
Fig 50 E-s ns E 60 days after axotomy

E 600 *sensitive Non specific Esterase* Three days after the operation the activity of this enzyme had definitely decreased in the ganglion cell cytoplasm (Figs 45 46 and 47) The maximum effect was obtained by the 5th day at which time the activity was very weak (Fig 48) After 30 days the reaction as only a little weaker than in the control ganglia (Fig 49) and 60 days after axotomy normalization was complete (Fig 50) By this time the morphological changes observed had also become completely normal

E 600 *resistant Non specific Esterase* The changes were similar to those observed in the case of E s ns E During the axon reaction the active cytoplasmic granules were less numerous than in the control section and the interstitial heavily stained spots were also greatly diminished in number Both these reappeared during the regenerative period By the 60th day the activity both in the ganglion cells and in the interstitial tissue was totally normalized

SYMPATHECTOMY

No major changes in the activities of the esterases were detected in the ciliary ganglion of the rat after this type of operation However a loss of some reactive fibres or cells m well have escaped notice since most of the tissue was unaffected by th is on

DISCUSSION

ACETYLCHOLINESTERASE

The staining intensity for AChE was found to vary considerably between the individual neurones in the ciliary ganglion of the rat in the present study This finding is at variance with those of the previous investigations on other species (Koelle 1951 1955 Fukuda and Koelle 1959 Koelle and Koelle 1959 McIsaac and Koelle 1959 Cauna *et al* 1961 Taxi 1961, Fredricsson and Sjoqvist 1962, Okinaka *et al* 1963) in which all the ciliary ganglion cells were found to exhibit intense AChE activity even after a relatively short incubation time However in the present study only a few of the ciliary ganglion cells of the rat possessed a high activity while the majority of the neurones showed a weak to moderate reaction even after prolonged incubation time a process which only increases the intensity of the reaction but does not affect the distribution of the activity (Holmstedt and Sjoqvist 1959) The possibility that formalin fixation to which AChE is sensitive (Holmstedt and Sjoqvist 1961) was the cause can be excluded since a similar variation between the individual neurones could be seen in fresh sections as well The different results may partly be due to differences in technique but the most probable cause is that the ciliary ganglion cells of the rat are different from those of the other mammals studied

In the ciliary ganglion of the rat two types of neurones were found one exhibiting intense AChE activity and the other weak to moderate activity A similar differentiation in the AChE activity of individual neurones can be seen in some parasympathetic ganglia (Koelle and Koelle 1959 Cauna *et al* 1961) in sympathetic ganglia (Koelle 1951 1955 Holmstedt 1957 Giacobini 1960 Taxi 1961 Sjoqvist 1962 Holmstedt *et al* 1963 Harkonen 1964) and in sensory ganglia and other sensory neurones (Koelle 1951 1955 Giacobini 1960 Esila 1963 Kokko 1965) while the motor neurones usually exhibit a uniformly high AChE activity (Nachmansohn and Hoff 1944 Schwarzacher 1958 Giacobini 1960 Snell 1961 Soderholm 1965)

It has been suggested that the neurones with a high AChE activity are purely cholinergic and those with a low activity non cholinergic the latter possessing some transmitter other than ACh (Koelle 1955 Giacobini 1960) although ACh is presumed to have some function in adrenergic transmission too (Burn and Rand 1960 1965) This is supported by the investigation of Birks and MacIntosh (1961) The adrenergic and sensory neurones usually possess a low or variable AChE activity (Koelle 1955) The nature of those ciliary ganglion cells of the rat that exhibited a low AChE activity has not been determined in the present study although several pharmacological studies suggest that some kind of adrenergic synaptic transmission takes place in the ciliary ganglion of the cat and the dog (Tum Suden *et al* 1951 1952 Perry and Talesnik 1953) This view is supported by the present study (see Chapter IV)

Electrophoretically AChE is not homogeneous (Bernsohn *et al* 1962 Eranko *et al* 1964 Härkonen 1964 Kokko 1965 Soderholm 1965) and it is possible that its several forms which may have different functional characteristics are responsible for the variations observed in the staining intensity of the neurones This might also partly explain the differences in AChE activity in the rat ciliary ganglion cells

The synaptic terminals which were numerous and formed a continuous zone around the perikarya of some ganglion cells exhibited a higher activity than the neuronal cytoplasm These results are in keeping with previous studies of the ciliary ganglion cells of mammals and birds as well as of reptiles This activity is distributed in both pre and postsynaptic structures in various ganglia including the ciliary (Koelle 1951, 1955 Szentágothai *et al* 1955 Szentagothai 1957, Brown 1958 Koelle and Koelle 1959 Taxi 1961 Fredricsson and Sjoqvist 1962 Harkonen 1964) Biochemical determinations have verified this result (Giacobini 1960) The high AChE activity seen in the synapses suggests a cholinergic transmission in the ciliary ganglion of the rat although the demonstration of AChE alone cannot be taken as absolute proof of the presence of a cholinergic type of transmission (Giacobini 1960 Koelle 1963 Shute and Lewis 1966 Eranko 1967)

The AChE positive nerve fibres were mainly postganglionic axons as demonstrated by the results of the denervation procedures This finding is in good agreement with those of previous investigations (Koelle 1951 Gerebtzoff 1959 Taxi 1961) The capillaries in the ciliary ganglion of the rat did not show any AChE activity although active capillaries have been seen in the central nervous system (Crook 1963)

Decentralization had no effect on the cytoplasmic AChE activity, as was also seen in previous investigations concerning the ciliary ganglion (Szentá gothai *et al* 1955 Koelle and Koelle 1959 Taxi 1961) and other ganglia (Brown 1958 Snell 1958 Holmstedt *et al* 1963 Harkonen 1964) However the synaptic reactivity diminished somewhat after section of the oculomotor nerve This may be due to degeneration of the preganglionic synaptic structures upon loss of their enzyme activity This explanation fits well with the results of previous studies (Szentagothai *et al* 1955 Koelle and Koelle 1959 Taxi 1961) In sympathetic ganglia in addition to the synaptic changes disappearance of the preganglionic nerve fibres (Brown 1958 Snell 1958 Koelle and Koelle 1959, Harkonen 1964) and a marked decrease in the AChE concentration of the whole ganglion (v Brucke 1937 Dhar 1958 Harkonen 1964) were observed The number of reactive nerve fibres did not change in the present study after decentralization which establishes that preganglionic fibres are not AChE positive in the ciliary ganglion of the rat However the neurones of the Edinger Westphal nucleus from which the preganglionic nerves of the ciliary ganglion arise exhibited intense AChE activity (Koelle 1954 1955 a Gerebtzoff 1959)

Axotomy brought about many drastic changes in the enzyme activity of the ciliary ganglion In three days most of the AChE had disappeared so that only a few synapses and neurones still exhibited activity These ganglion

cells had possibly escaped axotomy High activity was seen in the proximal axon stump in some sections demonstrating the axonal flow of AChE as reported by other workers (Sawyer 1946 Harkonen 1964 Eranko and Harkonen 1965) Almost complete disappearance of the cytoplasmic axonal and postganglionic synaptic AChE activity has been described as taking place in other ganglia and motor neurones after axotomy (Sawyer and Hollinshead 1945 Dhar 1958 Schwarzacher 1958 Harkonen 1964 Gromadzki and Koelle 1965 Soderholm 1965) followed by functional changes while the production of ACh keeps within normal limits (Brown *et al* 1952 Brown and Pascoe 1954)

According to McLennan (1954) axotomy in the sympathetic ganglion of the cat had no effect on the AChE content of the preganglionic fibres and synaptic structures In the present study it was therefore surprising to see that most of the preganglionic synaptic terminals had lost their AChE activity after axotomy Similar changes have also been observed in some previous investigations (Harkonen 1964 Illis 1964 Soderholm 1965)

This change may be explained in several ways (1) During the operation the preganglionic nerve fibres may have been damaged by stretching or by deficiency of blood supply so that these fibres degenerated and lost their AChE activity (Taxi 1961) (2) Axotomy may really influence the AChE activity of the preganglionic synaptic structures (Sawyer and Hollinshead 1945 Brown 1958) The preganglionic neurone after it observes that the postganglionic neurone is unable to conduct impulses to the effector organ may stop producing AChE (3) The highly active apparently synaptic zone may in fact not contain any synapses at all but the reactivity may be due to activity in the endoplasmic reticulum concentrated in the periphery of the ganglion cell cytoplasm However the results of preganglionic denervation do not support this suggestion (4) The preganglionic neurones may have had some transmitter other than ACh so that AChE activity was not needed in the synapses either

These suggestions are highly speculative and the problem is still unsolved Electron microscopic investigations in combination with histochemical methods are necessary for its solution since many ultrastructural changes in the neuronal cytoplasm and synapses have been observed after nerve division (De Robertis 1956 Harkonen 1964 Hunt and Nelson 1965 Lewis and Shute 1965 a)

The changes in enzyme reactivity observed after nerve section were reversible for during the regenerative period activity was normalized However the length of time between the minimal (or maximal) enzyme activity and its subsequent complete return to normal varies This is probably due to distance of the denervation sites from the cell bodies (Kawai 1963 Brodal to be published)

In the present study ns ChE was found to be mainly localized in the interstitial tissue which consists of many tissue components satellite cells Schwann cells, pre and postganglionic axons dendrites and capillaries all of which apparently exhibited ns ChE activity although it was difficult to determine the exact site of the esterase activity These results agree well with findings concerning the ciliary ganglia of other mammals (Koelle 1951 1955 Giacobini 1959 1960 Cauna et al 1961 Taxi 1961) and also mammalian sympathetic and sensory ganglia (Koelle 1951 1955 Giacobini 1956 1959 Holmstedt 1957 Cauna et al 1961 Taxi 1961 Harkonen 1964 Kokko 1965) Some ciliary ganglion cells of the rat exhibited a strong ns ChE activity which has not been reported in any nerve cells of this ganglion in the other mammals studied (Koelle 1951 1955 Giacobini 1959 1960 Cauna et al 1961, Taxi 1961) A similar difference between the ganglionic neuronal ns ChE activity of the rat and that of other mammals has been encountered in the sympathetic and sensory ganglia (Koelle 1951 1955 Giacobini 1959 Cauna et al 1961 Taxi 1961, Harkonen 1964 Kokko 1965) Ns ChE activity is also present in the central nervous system where it is mainly localized in the glial cells myelinated nerve fibres and capillaries (Hard and Peterson 1949 Ord and Thompson 1952 Cavanagh et al 1954 Koelle 1954 1955 a Foldes et al 1962 Teoharov 1962) In addition to the nervous system all other tissues including the blood also contain ns ChE (Alles and Hawes 1940 Richter and Croft 1942 Aldridge 1954 Easson and Stedman 1957)

Since ns ChE is so widely distributed in various tissues it must play some role although this is not known Many hypotheses have been put forward Clitherow et al (1963) suggested that in the liver it might hydrolyse toxic byproducts of lipid metabolism In brain tissue ns ChE might be involved in γ aminobutyrylcholine metabolism (Holmstedt and Sjoqvist 1960) while in the synaptic structures it might eliminate any stray AChE (Koelle 1962) In the walls of the capillaries ns ChE might participate in the regulation of permeability (Koelle 1955 a) The high ns ChE activity observed in the satellite cells confirms the metabolic or other exchanges taking place between the neurone and its glia (Scharf 1958 Hydén 1960)

As a result of both pre and postganglionic denervation the interstitial ns ChE activity decreased remarkably in a few days and more strikingly after axotomy than after decentralization These results correspond well with those observed in the sympathetic ganglion after denervation (Harkonen 1964) The myelinated nerves as a rule contain ns ChE (Coupland and Holmes 1957) which is mainly localized in the myelin sheath (Cavanagh et al 1954 Tewari and Bourne 1960) Therefore the decreased activity observed in the present study after decentralization is readily understandable since this operation brings about the degeneration of myelin sheaths in the distal part of sectioned nerve fibres such as the sympathetic ganglion (Sawyer and Hollinshead 1945) The remaining activity was probably due

to that of postganglionic nerve fibres and satellite and Schwann cells which had not been affected by preganglionic denervation In degenerating peripheral nerves on the contrary ns ChE rose above the normal value (Cavanagh *et al* 1954)

Postganglionic denervation on the other hand should not cause any myelin degeneration in the ganglion Therefore the great decrease of ns ChE activity observed after axotomy must mainly be due to diminished reactivity in the glial cells which might partly result from some non specific factors such as ischaemia caused by the operation (Harkonen 1964) However axotomy may also affect the enzyme activity of the preganglionic structures (Sawyer and Hollinshead 1945) The satellite cells were affected much less than the Schwann cells while the normally positive ganglion cells had totally lost their activity after section of the short ciliary nerves

NON SPECIFIC ESTERASES

E s ns E exhibited an intense activity in practically all the ciliary ganglion cells of the rat and the variation between individual neurones was less than in the sympathetic (Savay *et al* 1953 Harkonen 1964) and spinal (Thomas 1963 Kokko 1965) ganglia The E r ns E activity was less intense than that of E s ns E and it was mainly localized in cytoplasmic granules as has also been shown to be the case in other ganglia (Harkonen 1964 Fisher and Sutherland 1965 a Kokko 1965) and spinal cord neurones (Soderholm 1965) There was also another difference between E s ns E and E r ns E namely that the latter could only be demonstrated in fixed sections while the former was most active in fresh sections and almost negative in fixed sections Even postfixation did not immobilize E r ns E Thus the desmo-enzyme nature of E s ns E and the lyo-enzyme nature of E r ns E were also confirmed in the present study Further several histochemical and starch gel electophoretic investigations concerning the nervous tissue have shown this view to be correct (Eranko *et al* 1962 1964 Harkonen 1964 Kokko 1965 Soderholm 1965)

E s ns E seems to be bound to the endoplasmic reticulum and is poorly soluble This enzyme however is very easily inactivated by formalin (Hannibal and Nachlas 1959) so that it is hardly demonstrable at all in fixed material E r ns E is mainly associated with granules (Harkonen 1964 Fisher and Sutherland 1965 Kokko 1965) an observation which has been supported by biochemical and electron microscopic studies (Aldridge and Johnson 1959 Torack and Barrnett 1962)

Ns Es are widely distributed in living tissues (Nachlas and Seligman 1949 Barrnett 1952 Gomori 1953 1955 Mendel *et al* 1953 Markert and Hunter 1959) and they behave in a great variety of ways in relation to substrates or other reagents (Barrnett and Seligman 1951 Bergmann *et al* 1957 Burstone 1957 Pearson and Defendi 1957 Underhay 1957 Bergmann and Rimon 1958) Therefore the concept ns E consists of a great variety of

enzymes or enzyme groups the functions of which are not exactly known Some authors have suggested that they are proteolytic enzymes in nervous tissue (Pearse 1956, Pepler and Pearse 1957 Hess and Pearse 1958) and also in other tissues (Hopsu and Glenner 1963 1964) while others believe that they hydrolyse lipids (Myers and Mendel 1953 Novikoff 1961) It has also been thought that ns Es may affect the growth and development of lower organisms but that in the course of evolution the latter significance of ns Es has been lost in higher animals (Myers and Mendel 1953)

Decentralization was found to have no effect on ns E activities in the ciliary ganglion of the rat while axotomy caused a marked, but in general, reversible decrease of both E s ns E and E r ns E activities The behaviour of the latter enzyme differs from that described by Harkonen (1964) and Fisher and Sutherland (1965 a) Many of the E r ns E positive granules had lost their enzyme activity after axotomy as observed by Soderholm (1965) and in the present study

In size shape and cytoplasmic distribution these granules, some at least of which give a positive acid phosphatase reaction (Koenig 1962 1964 Kawai 1963, Sharma 1964), resemble lipofuscin granules (Gedigk and Bontke 1956) lysosomes (De Duve *et al* 1955 Wachstein and Meisel 1960 Novikoff and Essner 1962 De Duve 1963, Novikoff 1963 Goldfischer *et al* 1964 Fisher and Sutherland 1965 a) or dense bodies (Torack and Barrnett 1962) Some granules are also autofluorescent (Kokko 1965) After axotomy however the E r ns E positive granules behave in a dissimilar way to the acid phosphatase positive ones since the latter increase after the operation (see Chapter II) Therefore all these granules cannot be identical

The decreased activity of ns Es after postganglionic denervation can be explained in the following way after axotomy anabolic processes are greatly intensified in the cell body (Brattgård *et al* 1957) and synthesizing enzymes, are needed while catabolic enzymes like hydrolases (e g esterases) are not Therefore the former increase and the latter decrease after axotomy (Soderholm 1965)

II PHOSPHATASES

EARLIER INVESTIGATIONS

NORMAL NERVOUS TISSUE

Acid Phosphatase

The author is unaware of any publication dealing with phosphatase activities in the ciliary ganglion but other nervous tissues have been studied A weak acid phosphatase (AcPase) reaction has been shown to occur in the stellate ganglion cells of the cat (Smith and Luttrell 1947 Smith 1948) while in the sympathetic ganglia of the guinea pig and man an intense staining has been observed (Walter 1955 Anderson and Son, 1962) In the spinal ganglion of the rat the small cells exhibited a stronger activity than the larger ones (Tewari and Bourne 1962 c Kokko 1965) in the trigeminal and nodose ganglia of the rat (Tewari and Bourne 1963 1964) and the monkey (Fisher and Sutherland 1965 a) the variation in AcPase activity between individual cells was not so great In the motor neurones of the spinal cord of the monkey (Bodian and Mellors 1945) the guinea pig (LaVelle *et al* 1954) and the rat (Soderholm 1965) a high activity has been reported

The distribution of AcPase is mainly cytoplasmic while the nuclear staining sometimes seen has been suggested to be an artifact (Eranko 1951 Barron and Sklar 1961 Deane 1963) Tewari and Bourne (1962) have reported AcPase in the synapses of the rat cerebellum and suggested that this enzyme might possess some function in synaptic transmission as well

Examined under a light microscope the neuronal AcPase seems to be localized in granules especially in fixed sections (Samorajski and Fitz 1961 Fisher 1965 Kokko 1965) and electron microscopic studies have confirmed these results (Novikoff 1963 Goldfischer *et al* 1964 Sobel and Avrin 1965) The AcPase-containing granules or dense bodies may be lysosomes some near the Golgi apparatus and others in the endoplasmic reticulum (Novikoff and Essner 1962 Koenig 1963 Novikoff 1963 Osinchak 1963 Sobel and Avrin 1965) It has also been thought that AcPase is manufactured in ribosomes and then transported via the Golgi apparatus to the lysosomes (Goldfischer *et al* 1964 Sobel and Avrin 1965) Some authors have reported that lipofuscin granules contain AcPase (Gedigk and Bontke 1956 Koenig 1964) while Barka (1962) suggested that the AcPase-positive structures may have been derived from pinocytotic processes

Alkaline Phosphatase

In a study on the sympathetic ganglion of the guinea pig and man Walter (1955) reported a strong alkaline phosphatase (AlPase) activity in the capillaries the nerve fibres the connective tissue and elsewhere in the perineuronal tissue while the ganglion cell cytoplasm was devoid of activity with the exception of the nuclear membrane and some fibrils within the nuclei In the semilunar ganglion of the cow the ganglion cell cytoplasm showed a reaction in fresh sections which varied from strong to negative (Scharf and Rowe 1957) The interstitial tissue was also intensely reactive In the spinal and trigeminal ganglia of the rat a diffuse perineuronal activity has been reported in addition to the strong reaction in the capillaries in fresh and fixed sections (Tewari and Bourne 1962 c 1963 1964)

In the rat brain and spinal cord capillaries have usually been reported to be the only active sites (Becker *et al* 1960 Fishman and Havash 1962 Soderholm 1965) but Tewari and Bourne (1962 1963 c) and Nandy and Bourne (1963) have found AlPase activity in the synaptic regions as well In the rabbit spinal cord nerve cell processes and glial nuclei stained positively (Samorajski and Fitz 1961)

Adenosine Triphosphatase

In the spinal and trigeminal ganglia of the rat Tewari and Bourne (1962 c 1963 1963 a) have reported a positive reaction for adenosine triphosphatase (ATPase) in the nerve cells in addition to intense activity in the nerve fibres and capillaries The activity varied considerably between individual ganglion cells Some activity was also seen in the nuclei and nucleoli (Sandler and Bourne 1962)

In the spinal cord of the rat and the guinea pig ATPase is said to be mainly confined to the neurones (the cell membrane the mitochondria the endoplasmic membranes the nuclei and the nucleoli) and capillaries (Wawrzyniak 1963 Nandy and Bourne 1964) However Soderholm (1965) did not observe any intracytoplasmic reaction in the anterior horn cells of the rat spinal cord In the rat brain ATPase is distributed in much the same way as in the spinal cord (Becker *et al* 1960 McClurkin 1964 Torack 1965) Naidoo (1962) has emphasized the high nuclear activity No synaptical activity has been reported in the rat cerebellum (Tewari and Bourne 1963 b)

With the aid of the electron microscope ATPase has been localized in the glial neuronal contact areas at the surface of neurones and at the distal synaptic regions of the axon as well as in the glial processes adjacent to the walls of the capillaries Within the neurones the most prominent sites of activity of this enzyme were the Golgi apparatus and the proximal parts of the axon (Torack and Barrnett 1963) In myelinated nerve fibres ATPase activity was confined exclusively to the axoplasm (Torack 1965) In other tissues such as the liver intestine musculature etc ATPase was concentrated in the mitochondria (Lazarus and Barden 1962 Goldfischer *et al* 1964 Schulze and Wollenberger 1965) As determined biochemically the glial cells exhibited a higher ATPase activity than the nerve cells in Deiters nucleus in the rabbit the neurones however exhibited a high activity just inside the cell membrane (Cummins and Hydén 1962 Hyden 1962) Germain and Proulx (1965) have isolated synaptic vesicles containing ATPase from the rat brain

EFFECT OF DENERVATION

Preganglionic Denervation

There is no report available concerning the effect of preganglionic nerve division on the activities of the phosphatases Such a study is of obvious interest in view of the reports claiming synaptic localization of phosphatases (Tewari and Bourne 1962 1963 c Nandy and Bourne 1963)

Postganglionic Denervation

Acid Phosphatase There are numerous histochemical investigations in which an increase in AcPase activity has been reported in the motor neurones of the spinal cord of several species after section of the sciatic nerve (Bodian and Mellors 1945 Lassek and Bueker 1947 Smith 1948 Bueker *et al* 1949 Barron and Sklar 1961 Barron and Tuncbay 1962 Kawai 1963 Soderholm 1965) Samorajski and Fitz (1961) are the only authors who did not observe any such histochemically demonstrable change in the AcPase reaction of the rabbit spinal cord An increase of activity after axotomy has also been observed in the facial nuclei of the rabbit (Coimbra and Tavares 1964) in the hypoglossal nuclei of the cat (Barron and Sklar 1961) and in the dorsal vagal ganglia of the monkey (Fisher and Sutherland 1965 a) Similar changes have been demonstrated in the stellate and nodose ganglia of the cat after postganglionic denervation (Smith and Luttrell 1947 Smith 1948)

Biochemical determinations showed increased activity in the grey matter of the axotomized spinal cord of the rabbit to about three times the normal value while the activity in the white matter did not change significantly (Samorajski and Fitz 1961) On the other hand Fieschi and Soriani (1959) found *no* change in the AcPase activity in the spinal cord of the guinea pig after bilateral section of the sciatic nerve

The increased AcPase activity after axotomy was reversible in all the cases studied

Alkaline Phosphatase A lowered AlPase activity has been established biochemically in the spinal cord of the guinea pig and of the rabbit after sciatic nerve section (Fieschi and Soriani 1959 Samorajski and Fitz 1961) while no histochemical changes were seen in the spinal cord of the rabbit (Samorajski and Fitz 1961) or the rat (Soderholm 1965) after axotomy

Adenosine Triphosphatase A few days after unilateral sciatic nerve section the anterior horn cells of the rat spinal cord exhibited a strongly increased reaction at the cell membrane the activity returning to normal in about two months (Soderholm 1965) Biochemically a reduction in ATPase concentration has been detected in the spinal cord of the guinea pig after bilateral sciatic nerve division (Fieschi and Soriani 1959)

METHODS

Fresh [illegible] and fixed sections were used In the case of ATPase postfixation was performe[illegible] formol-calcium for 5 10 minutes at -4 to 0°C (Wachstein *et al* 1967) I[illegible] outine work only postfixed sections were used

Gomori's (1952) methods for acid and alkaline phosphatases were employed a mixture of equal parts of sodium α and β-glycerophosphate being used as substrate The incubation time was 60 minutes for AcPase and 30 minutes for AlPase at 37°C

ATPase was demonstrated with the method described by Wachstein and Meisel (1957) The incubation time was from 6 to 10 minutes at 37°C

Controls were made by incubating the sections in a solution from which the substrate was omitted Controls of AcPase were also made by incubating the sections in the usual solution to which 10^{-3}M sodium fluoride was added (Newman *et al* 1950) All these controls were always negative

RESULTS

NORMAL GANGLION

Acid Phosphatase

This enzyme was best demonstrated in postfixed sections because with fixed sections a non specific precipitate which could not be rinsed off was found all over the section The activity was mainly distributed in the ganglion cell cytoplasm and was partly diffuse and partly granular The diffuse activity was weak and evenly distributed throughout the cytoplasm The active coarse granules on the other hand were mainly accumulated perinuclearly sometimes in clumps although an even distribution was also seen in some neurones The nucleoli were sometimes positive but in the majority of ganglion cells the nuclei were totally devoid of activity A few satellite cells also exhibited some activity which was weaker however than in the neurones In the interstitium some activity could be seen in a few sections it was probably located in the Schwann cells (Figs 51 and 53)

In fresh sections the activity was mostly diffuse so that only blurred granules could be seen The interstitial structures exhibited a higher activity than that found in postfixed sections

Alkaline Phosphatase

The capillaries were intensely stained Some black granular deposits were also seen at the periphery of some of the ganglion cells (Fig 57) When the incubation time was prolonged to two hours a continuous black ring was observed round the ganglion cell bodies but the reaction was so diffuse that it was impossible to localize the activity exactly The nuclei and nucleoli were negative

The results were similar in fresh postfixed and fixed sections



Fig 51 Acid phosphatase Incubation time 60 min Postfixed section x 420
Fig 52 Adenosine triphosphatase Incubation time 6 min Postfixed section x 420

Adenosine Triphosphatase

A weak finely granular reaction was observed in the peripheral parts of the cytoplasm of some of the ganglion cells and this reaction was somewhat intensified by prolonged incubation Surrounding the ganglion cell bodies a strongly positive zone was seen It was very difficult however to localize this activity more exactly it may partly be due to that of the cell membranes partly to that of cell processes or synaptic structures or satellite cells Nerve fibres and glial cells exhibited an intense activity as did also the capillaries which were the components most intensely stained The nuclei and nucleoli were totally negative (Figs 52 and 59)

A similar distribution pattern of enzyme activity was observed in fresh and postfixed sections but in the former the reaction product was more diffuse than in the latter

PREGANGLIONIC DENERVATION

No distinct changes were observed in the activities of any of the phosphatases studied after decentralization in the ciliary ganglion of the rat although it seemed as if the ATPase activity decreased slightly after the operation However this change was not significant

Acid Phosphatase On the 5th postoperative day AcPase showed a somewhat increased activity in the neurones (Figs 53 and 54) The maximum intensity of the reaction was reached in 10 days (Fig 55) after which the activity remained for a fortnight at this high level The active cytoplasmic granules seemed to be more numerous and to stain more intensely than those of the control ganglion In some neurones these particles had mostly accumulated in the axon hillock and the corresponding axon exhibited a strong reaction for some distance The interstitial tissue showed no change in its activity after axotomy

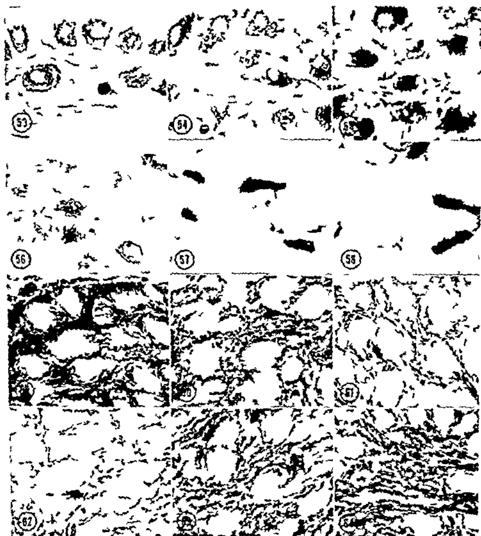
One month after neurotomy the reaction was still slightly stronger than in the control but by the 60th day the intensity of activity and the distribution and number of the positive granules were the same as in the contralateral ganglion (Fig 56)

Alkaline Phosphatase No significant changes were observed in the activity of this enzyme in the ciliary ganglion of the rat after axotomy (Figs 57 and 58)

Adenosine Triphosphatase Five days after postganglionic denervation the ATPase activity in the ganglion had slightly decreased (Figs 59 and 60) and the minimum level was reached by 10 to 15 days At this time the interstitial activity was weak with the exception of the capillaries which still gave an intense reaction The persisting activity seemed to be due to that of the ganglion cell membrane and of the satellite and glial cells in addition to the capillaries Thus only the nerve fibres had lost most of their ATPase (Figs 61 and 62) By the 30th postoperative day the interstitial activity was somewhat restored (Fig 63) and by the 60th day the reaction had totally normalized (Fig 64)

SYMPATHECTOMY

No clearly discernible changes were observed in the phosphatase activities in the ciliary ganglion of the rat after extirpation of the ipsilateral superior cervical ganglion



The effect of postganglionic denervation on phosphatase activities Incubation time 60 min for AcPase 30 min for AlPase and 6 min for ATPase Postfixed sections x 250

Fig 53 AcPase Control ganglion
Fig 56 AcPase 60 days after axotomy
Fig 59 ATPase Control ganglion
Fig 62 ATPase 15 days after axotomy

Fig 54 AcPase 5 days after axotomy
Fig 57 AlPase Control ganglion
Fig 60 ATPase 5 days after axotomy
Fig 63 ATPase 30 days after axotomy

Fig 55 AcPase 10 days after axotomy
Fig 58 AlPase 15 days after axotomy
Fig 61 ATPase 10 days after axotomy
Fig 64 ATPase 60 days after axotomy

DISCUSSION

ACID PHOSPHATASE

There was a marked difference in the cytoplasmic distribution of AcPase in fresh and fixed sections In the former the reaction product was so diffuse that granules could hardly be seen In postfixed sections however although these did sometimes also exhibit evidence of slight diffusion and in fixed sections the activity was mainly concentrated in granules Thus we are again faced with the question of whether AcPase is partly lyo and partly desmo-enzyme as was theoretically proposed by Lison (1948) and actually shown by Eranko (1952) Nachlas *et al* (1956) Hannibal and Nachlas (1959) and Kokko (1965) It is impossible however to solve this problem by histochemical methods alone In the living organism AcPase may be localized exclusively within granules but the many technical procedures before and during staining may rupture these particles and release the enzyme into the soluble cytoplasm (Bitensky 1963 De Duve 1963 Tappel *et al* 1963) Otherwise basically the results observed accord well with those of previous studies on other ganglia

Decentralization had no effect on the AcPase activity in the ciliary ganglion of the rat since the main activity was cytoplasmic so that degeneration of the preganglionic fibres did not change the general impression gained

The changes observed in AcPase activity in the axotomized ciliary ganglia are in keeping with the results of previous investigations concerning other neuronal tissue

The axon reaction causes an increase in the protein synthesis of the cell body (Brattgard *et al* 1957) for which phosphate derivatives are required these are most easily supplied by increasing the phosphatase activity (LaVelle *et al* 1954) A high AcPase activity has in fact been demonstrated in the secretory cells (Eranko 1951 Novikoff 1961 Sobel and Avrin 1965) suggesting that this enzyme is involved in synthetic processes

On the other hand Fisher and Sutherland (1965 a) have observed that an initial increase is followed by a decrease in AcPase activity in the neurones of the nodose ganglion and the dorsal vagal nuclei of the monkey According to these workers AcPase has a catabolic effect since the initial increase in the activity of the enzyme coincides well with chromatolysis whereas a decreased activity was observed during the recovery period at *which time chromosynthesis is taking place In the present study no such* lowered activity was seen

ALKALINE PHOSPHATASE

A high AlPase activity was observed exclusively in the capillaries of the rat ciliary ganglion as in other nervous tissue (Becker *et al* 1960 Fishman and Hayashi 1962 Bannister and Romanul 1963 Soderholm 1965)

Neither pre nor postganglionic denervation had any effect on the AlPase

activity in the ciliary ganglion of the rat This fits in well with previous histochemical studies dealing with the spinal cord (Samorajski and Fitz 1961 Soderholm 1965) The existence of normal activity in the capillaries of the denervated ganglia established that the blood supply had not been seriously disturbed if at all by the operations

ADENOSINE TRIPHOSPHATASE

ATPase in the ciliary ganglion cells was mainly localized in the cell membrane as has previously been seen in other neurones (Becker *et al* 1960 Cummins and Hyden 1962 Soderholm 1965) although some positive intracytoplasmic particles which may have been mitochondria or endoplasmic structures containing ATPase (Goldfischer *et al* 1964 Nandy and Bourne 1964) were also seen The cytoplasm of most ganglion cells was negative which may have been due to inhibition of the enzyme since ATPase is extremely sensitive (Myers and Slater 1957 Wachstein *et al* 1962)

The most important function of ATPase is to release energy by hydrolysing adenosine triphosphate (ATP) and so to furnish the energy required for the ion pump and thus for active transport as a rule across cell membranes (Cummins and Hyden 1962 Tewari and Bourne 1962 c 1963 b) Therefore a high ATPase activity is necessary at the surface of the capillary glial and neuronal cell membranes The propagation of nerve impulse involves the movements of ions across the conductive membrane and the energy required to restore the ionic imbalance of the quiescent membrane can be obtained by hydrolysing ATP Thus axonal dendritic and synaptic ATPase activity becomes explicable (Hyden 1962)

After axotomy the interstitial ATPase activity weakened considerably in the ciliary ganglion of the rat and the decrease can be explained as a consequence of axonal flow of the enzyme since it has been reported that the axoplasm contains ATPase (Torack 1965) However this explanation is highly speculative Increased activity as seen in the motor neurones of the spinal cord after sciatic neurotomy (Soderholm 1965) was not encountered in the present study

III OXIDATIVE ENZYMES

EARLIER INVESTIGATIONS

NORMAL NERVOUS TISSUE

Dehydrogenases

NADH and NADPH Tetrazolium Reductases As regards NADH (reduced nicotinamide adenine dinucleotide or reduced diphosphopyridine dinucleotide DPNH) and NADPH (reduced nicotinamide adenine dinucleotide phosphate or reduced triphosphopyridine dinucleotide TPNH) tetrazolium reductases otherwise known as DPN and TPN-diaphorase respectively a wide variation in the intensity and intracellular distribution of activity has been shown to exist between individual neurones in the sympathetic and sensory ganglia of mammals A positive reaction is demonstrable in all the ganglion cells especially in their perikarya The activity is usually stronger in them than in the neuropil in which it is mainly localized in the satellite Schwann and glial cells while the axoplasm exhibits only weak activity As a rule DPN-diaphorase gives a more intense reaction than TPN-diaphorase especially in the neuronal cytoplasm The nuclei and nucleoli are devoid of activity (Potanos *et al* 1959 Romanul and Cohen 1960 Szmorajski 1960 Thomas and Pearse 1961 Harkonen 1964)

A very similar distribution pattern is found in the spinal cord (Szmorajski 1960 Kumamoto and Bourne 1963 Soderholm 1965) and brain tissue (Becker *et al* 1960 Thomas and Pearse 1961 Friede and Fleming 1962 Rubinstein *et al* 1962) of mammals

In the pre and postsynaptic structures in the spinal cord of the rat Nandy and Bourne (1964 b) have demonstrated DPN-diaphorase positive granules while Soderholm (1965) did not observe any such reaction

NAD and NADP linked Tetrazolium Reductases NAD (nicotinamide adenine dinucleotide) and NADP (nicotinamide adenine dinucleotide phosphate) linked tetrazolium reductases generally called dehydrogenases are enzymes concerned in carbohydrate protein and fat metabolism

In the ciliary ganglia of birds and reptiles succinate dehydrogenase has been described to occur as intensely stained granules in the neuronal cytoplasm while in the submandibular ganglion of the rat the Schwann cells possess the highest activity No synaptic activity for the enzyme was seen in these ganglia (Szentágothai 1957) However in the synaptic areas of the spinal cord of the rat succinate dehydrogenase positive granules have been seen (Nandy and Bourne 1964 b) but Soderholm (1965) did not observe any

In ganglion cells and in other neurones glutamate alcohol succinate and α glycerophosphate dehydrogenases show weak activity while satellite cells and neuroglia exhibit strong reactions in tests for succinate and α glycerophosphate dehydrogenases (Hess and Pearse 1961 Kumamoto and Bourne 1963 Harkonen 1964 Ferguson 1965 Soderholm 1965) The reactivity of the latter enzyme can be intensified if menadione (2 methyl 1 4 naphtoquinone) is used as intermediate electron acceptor (Hess and Pearse 1961)

As to other dehydrogenases such as isocitrate lactate and malate tetrazolium reductases ganglionic tissue gives a positive reaction which is stronger in the nerve cell bodies than in the neuropil Such a picture has been described in the sympathetic ganglion of the rat (Harkonen 1964) In the sensory ganglia of mammals the small ganglion cells have usually been found to possess higher dehydrogenase activity than the larger ones neuroglia exhibits only weak activity (Scharf and Rowe 1957 Klein 1960 Romanul and Cohen 1960 Thomas and Pearse 1961 Tewari and Bourne 1963)

In the spinal cord of the rat (Nandy and Bourne 1964 a Soderholm 1965) and in the brain tissue of mammals (Padykula 1952 Robins and Smith 1953 Mustakallio 1954 Shimizu and Morikawa 1957 Shimizu *et al* 1957 Friede 1960 Friede and Fleming 1962 Tewari and Bourne 1962) the grey matter as a rule exhibits strong dehydrogenase activity compared with that of the white matter which however also possesses some activity (Wolfgram and Rose 1959)

Biochemical studies of the rabbit brain have demonstrated a higher succinate dehydrogenase content in the white matter than in the grey (Kawakita 1956) with a microgasometric technique corresponding results have been obtained concerning the distribution of this enzyme in the spinal ganglion of the rabbit (Hyden *et al* 1958) These results thus support the histochemical findings However glutamate dehydrogenase exhibits a higher activity than malate dehydrogenase in the neurones of Deiters nucleus in the rabbit while the histochemical picture is the opposite (Hamberger 1961)

Oxidases

Cytochrome Oxidase (CytO) This enzyme formerly known as indophenol oxidase has been described as giving a positive and mainly granular reaction in the perikarya and synaptic regions in the ciliary ganglia of birds and reptiles and in the submandibular ganglion of the rat (Szentágothai 1957) CytO positive granules have also been encountered in the synapses of the spinal cord of the rat (Nandy and Bourne 1964 b)

In all the sympathetic ganglion cells of the rat a positive reaction has been observed the capsular cells and other interstitial tissue exhibited weaker activity (Harkonen 1964) In the trigeminal ganglion of the newborn rat an intense reaction was observed in all the ganglion cells (Burstone 1961) while in the spinal ganglion of the rat activity varied between individual neurones from weak to intense (Tewari and Bourne 1962 a)

In the brain tissue the grey matter possesses strong activity while the white matter is almost negative (Meath and Pope 1950 Shimizu *et al* 1957 Burstone 1959 Hannibal *et al* 1960) The activity is especially intense in the neuronal cell bodies dendritic processes and axonal terminals although in the cerebellum of the rat Tewari and Bourne (1962) have demonstrated strong activity in the white matter too

Monoamine Oxidase (MAO) In the ciliary ganglion of the cat the perikarya were all stained with varying intensity but granularity was weak In addition to the granular reaction a homogeneous purplish background staining was observed in the cytoplasm of the nerve cells Nerve fibres and their terminations also exhibited similar background plus granular staining while other interstitial structures were negative In the rabbit the ciliary ganglion cells were only faintly stained (Koelle and Valk 1954)

A strong granular and weak diffuse MAO activity has been described in the sympathetic and sensory ganglion cells of the cat (Koelle and Valk 1954) the guinea pig (Glenner *et al* 1957) and the rat (Harkonen 1964 Klingman and Klingman 1966) This activity was less intense in the rabbit than in the cat tissue (Koelle and Valk 1954) The ganglion cells of Auerbach s and Meissner s plexuses also exhibited a high activity (Eder 1957 Wohlrab 1961) In the central nervous system of rodents MAO has a mainly neuronal distribution (Arioka and Tanimukai 1957 Shimizu *et al* 1959) although Tewari and Bourne (1963 d) described a stronger reaction in the white matter than in the grey in the rat cerebellum

Intracellular Distribution of Oxidative Enzymes

From histochemical studies it can be seen that both dehydrogenases and oxidases are partly localized in cytoplasmic granules which have been assumed to be mitochondria (Hughes 1956) Friede and Pax (1961) established this for succinate dehydrogenase and CytO while Walker and Seligman (1963) added to this group β-hydroxybutyrate lactate malate and isocitrate dehydrogenases Many cytochemical investigations have established that enzymes of the tricarboxylic acid cycle in addition to those of the cytochrome system and to β-hydroxybutyrate dehydrogenase are associated with mitochondria (Siekevitz and Watson 1956 Pearse *et al* 1958 Scarpelli and Pearse 1958) Becker *et al* (1960) suggested that DPN-diaphorase is located in mitochondria and ergastoplasm whereas TPN-diaphorase is only present in mitochondria These results are supported by Vesco and Giuditta (1966)

Biochemical studies have also demonstrated that both CytO and MAO are located in mitochondria (Brody *et al* 1952 Hogeboom and Schneider 1952 Blaschko *et al* 1957 Weiner 1960)

Electron microscopically NADH tetrazolium reductase and succinate dehydrogenase are seen to be localized exclusively in mitochondria (Ogawa and Barrnett 1965)

EFFECT OF DENERVATION

Preganglionic Denervation

No change was observed after decentralization in either the dehydrogenase or oxidase activities of the superior cervical ganglion of the rat (Harkonen 1964)

Postganglionic Denervation

Dehydrogenases An increase in all the dehydrogenase activities was seen in the sympathetic ganglion cells of the rat after axotomy The change was as a rule reversible The activities of dehydrogenases were concentrated in a central region of the axotomized neurone (Harkonen 1964) In the spinal ganglion of the rat increased succinate dehydrogenase activity was seen after sciatic nerve section and at the same time the activity of the enzyme was intensified in the proximal stump of the axon indicating axonal flow of the enzyme (Klein 1960)

In the motor neurones of the rat spinal cord the succinate and NADPH tetrazolium reductase activities diminished after axotomy (Friede 1959) while in the guinea pig Kumamoto and Bourne (1963) observed a slight increase in DPN-diaphorase activity and a decreased succinate dehydrogenase activity in both the spinal cord and ganglion Soderholm (1965) described an increase in the activities of dehydrogenases in the anterior horn cells of the rat spinal cord after axotomy with the exception of α glycerophosphate menadione and succinate tetrazolium reductase the activities of which did not change TPN-diaphorase and a few dehydrogenases exhibited increased activity in the neurones of the facial nuclei of the guinea pig (Kreutzberg 1963) and the rabbit (Fisher and Malik 1964) after division of the ipsilateral facial nerve

Biochemically succinate dehydrogenase activity has been demonstrated to decrease somewhat in the axotomized spinal cord of the monkey (about 12 %) (Howe and Flexner 1947) and the guinea pig (about 7 %) (Kumamoto and Bourne 1963) In the latter species a greater decrease (about 15 %) was observed in the spinal ganglion while lactate dehydrogenase did not undergo any significant change On the other hand Harkonen (1964) demonstrated an increase in the lactate and glucose-6 phosphate dehydrogenase activities in the sympathetic ganglion of the rat after axotomy

Oxidases Histochemically decreased CytO activity after axotomy has been established in the sympathetic ganglion cells and in the interstitial structures of the rat (Harkonen 1964) and also in the spinal cord of the guinea pig (Kumamoto and Bourne 1963) The number of active granules diminished in the nerve cells These results coincide well with those of biochemical studies concerning the thalamic nuclei and the spinal cord of the cat and the monkey after decortication and axotomy respectively (Howe and Mellors 1945) However the latter workers did not observe any temporal correlation between chromatolysis and CytO concentration Therefore they suggested that the decrease in the CytO content was due to degeneration of some neurones rather than to the axon reaction

After axotomy MAO activity decreased considerably in the superior cervical ganglion of the rat although an initial increase was observed This enzyme also exhibited axonal flow (Harkonen 1964)

Dehydrogenases

NADH and NADPH Tetrazolium Reductases The method described by Scarpelli *et al* (1958) was used NADH and NADPH were used as substrates and nitro blue tetrazolium salt (2 2-di p-nitrophenyl 5,5-diphenyl 3,3 [3,3-dimethoxy-4 4 biphenylene] ditetrazolium chloride) abbreviated NBT was used as final electron acceptor The incubation time was 30 minutes for DPN-diaphorase and 60 minutes for TPN-diaphorase at 37 C

NAD linked Tetrazolium Reductases The method described by Hess *et al* (1958) was employed The substrates used were β-hydroxybutyrate isocitrate malate glutamate lactate α glycerophosphate and ethanol NBT was used as final electron acceptor The incubation time was 60 minutes for glutamate and alcohol dehydrogenase and 30 minutes for the other enzymes at 37°C

NADP linked Tetrazolium Reductases Glucose 6-phosphate as substrate and NBT as final electron acceptor were used to demonstrate glucose-6-phosphate dehydrogenase according to the method of Hess *et al* (1958) The incubation time was 45 minutes at 37°C

α Glycerophosphate Menadione Tetrazolium Reductase Sodium α glycerophosphate as substrate menadione as co-factor and NBT as electron acceptor were used to demonstrate this enzyme according to the method described by Wattenberg and Leong (1960) and Wattenberg (1961) The incubation time was 30 minutes at 37 C

Succinate Tetrazolium Reductase Sodium succinate as substrate and NBT as final electron acceptor were used to demonstrate this enzyme according to the method of Nachlas *et al* (1957) The incubation time was 45 minutes at 37°C The effect of phenazine methosulphate (PMS) in a concentration of 30µg/ml of the incubation medium was studied as well

A study was also made of the effect of 5 minutes postfixation in cold acetone on the intracytoplasmic distribution of various dehydrogenases The sections were well rinsed in distilled water before incubation (Novikoff *et al* 1960)

The controls were made in three ways (1) The sections were incubated in a medium from which the substrate was omitted and the endogenous substrates were washed away by preincubation in a buffer solution for 5 minutes (2) Co-enzyme or in the case of succinate dehydrogenase NBT was omitted from the incubation solution (3) The enzymes were inactivated by heating the sections for 3—5 minutes at 80 C in distilled water before incubation These controls were always negative

Oxidases

Cytochrome Oxidase This enzyme was demonstrated according to Burstone's method (1959 1960 1961) as modified by Harkonen (1964) The incubation time was 45—60 minutes at room temperature

Controls were made by omitting the substrate from the incubation solution or by destroying the enzyme by heating as in the case of dehydrogenases These controls were totally devoid of activity When potassium cyanide in a concentration of 10^{-3}M was added to the incubation medium the activity was also totally inhibited

Monoamine Oxidase Both the method described by Koelle and Valk (1954) and that presented by Glenner *et al* (1957) were used to demonstrate this enzyme The former method as modified by Harkonen (1964) was used routinely because of the superior intensity of the reaction The incubation time was 2 hours at 37°C

Controls were made by incubating the sections without substrate or in the presence of 10 M Marsilid (isonicotinyl 2 isopropyl hydrazine phosphate) in both the preincubation and incubation [illegible] These controls were totally negative

RESULTS

NORMAL GANGLION

Dehydrogenases

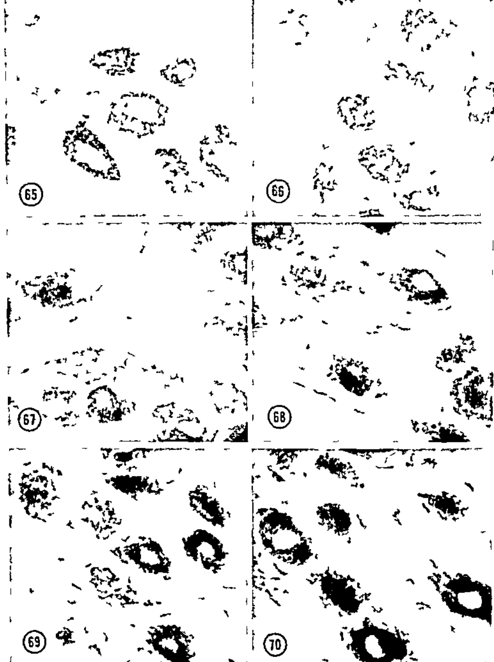
NADH and NADPH-Tetrazolium Reductases *DPN diaphorase* activity was moderate to intense in the ciliary ganglion cells between which some variation in the intensity of staining was observed All the neurones were positive Both diffuse and granular activity were seen The particles were usually evenly distributed throughout the whole cytoplasm but in some nerve cells they had accumulated in the central parts The diffuse staining in the ganglion cell cytoplasm was even and weak It was difficult to determine with certainty whether there was really any activity at the sites of the synapses In the satellite cells a few large granules could be seen their distribution being mainly perinuclear However the reactivity of the capsular cells was much weaker than that of the ganglion cells In the neuropil only a few positive granules were seen in addition to the very weak interstitial background staining the particulate activity was probably localized in the Schwann and glial cells The nuclei and nucleoli were totally devoid of activity (Figs 65 and 77)

TPN diaphorase activity differed somewhat from that of the preceding enzyme The intensity of the neuronal cytoplasmic reaction was weaker than that of DPN-diaphorase in spite of the longer incubation time The mean size of the positive granules was larger and their number less than in the case of DPN-diaphorase The interstitial structures exhibited an equally strong reaction as for DPN-diaphorase The nuclei and nucleoli were negative (Fig 66)

NAD^+ linked Tetrazolium Reductases Moderate *β hydroxybutyrate dehydrogenase* activity was localized mainly in small granules but there was also a weak diffuse cytoplasmic staining The particles were mostly concentrated in the central regions of the neurones A slight reaction was also observed in the nuclei and nucleoli The cytoplasmic activity varied somewhat between individual nerve cells The other tissue components exhibited both granular and diffuse activity higher than that of DPN diaphorase (Fig 67)

Isocitrate malate and lactate tetrazolium reductases all exhibited strong activity in all the ciliary ganglion cells In addition to the weak diffuse cytoplasmic staining intensely positive particles were observed which in most neurones were distributed around the unstained nuclei These enzymes showed the highest dehydrogenase activity in the ciliary ganglion cells of the rat The interstitial tissue components were stained as with DPN diaphorase (Figs 68 69 and 70)

Glutamate tetrazolium reductase exhibited only a weak and mainly granular activity in all the neurones of the ciliary ganglion Nucleoli in the otherwise unstained nuclei were positive in some neurones The interstitial tissue including the satellite cells was also weakly stained (Fig 71) No



Oxidative enzyme activities Fresh sections x 420

Fig 65 NADH tetrazolium reductase NBT incubation time 30 min
Fig 67 β-Hydroxybutyrate tetrazolium reductase NBT incubation time 30 min
Fig 69 Malate tetrazolium reductase NBT incubation time 30 min

Fig 66 NADPH tetrazolium reductase NBT incubation time 60 min
Fig 68 Isocitrate tetrazolium NBT incub 30
Fig 70
NBT 30



Oxidative enzyme activities Frech sections x 420

Fig 71 Glutamate tetrazolium reductase NBT incubation time 60 min
Fig 73 α-Glycerophosphate tetrazolium reductase NBT incubation time 30 min
Fig 75 Cytochrome oxidase Incubation time 60 min

Fig 72 Glucose 6-phosphate tetrazolium reductase NBT incubation time 45 min
Fig 74 Succinate tetrazolium reductase NBT incubation time 45 min
Fig 76 Monoamine oxidase (Koelle Valk) Incubation time 2 hours

positive reaction for *alcohol dehydrogenase* could be detected in the cells of the ciliary ganglion However all over the sections discrete granules were seen which were probably due to the lipid solubility of the tetrazolium salt used If the incubation time was prolonged for 2—3 hours no further activity was observed with either of these two enzymes

NADP⁺ linked Tetrazolium Reductase The only representative of this group in the present study *glucose 6 phosphate dehydrogenase*, exhibited a moderate activity in all the ganglion cells Most of the activity was diffuse the granules were less numerous and were evenly distributed throughout the cytoplasm Interstitial structures were stained as in the case of other dehydrogenases The nuclei were totally inactive (Fig 72)

α Glycerophosphate Menadione Tetrazolium Reductase A moderate reaction was seen in all the ganglion cells The granules were evenly distributed throughout the cytoplasm The diffuse activity was weak The nuclei were devoid of any activity Around a few neurones a granular precipitate was found which in some sections was very intense This reaction was probably nonspecific rather than synaptic because these granules were also of a different colour than those in the neuronal cytoplasm The interstitial tissue showed the weak reaction (Fig 73) If NAD was substituted for menadione a weaker reaction was obtained in the ganglion cells

Succinate tetrazolium reductase was strongly positive in the ganglion cells and mainly granular although a weak diffuse cytoplasmic background staining was also seen The granules were mainly perinuclearly distributed The nuclei were totally unstained Other components of the ganglion exhibited a reaction similar to that of DPN diaphorase (Figs 74 and 89) The addition of PMS to the incubation solution enhanced the reactivity without affecting the quality or distribution of the reaction product

After postfixation in cold acetone the diffuse cytoplasmic reaction of the dehydrogenases studied was less strong than in the fresh sections and so rendered the picture sharper The granular activity was unchanged

Oxidases

Cytochrome oxidase All the neurones were moderately stained and some activity was also seen to extend into the cell processes The staining of the individual cells varied somewhat in intensity The distribution of the granules which were numerous was mostly perinuclear A weak diffuse staining was observed in the cytoplasm of the ganglion cells The satellite cells and neuropil exhibited a stronger activity than in the case of the dehydrogenases but weaker than that in the nerve cell bodies Some of the nerve fibres were intensely stained these were probably myelinated The weak interstitial activity was partly granular and partly diffuse The nuclei and nucleoli were negative (Figs 75 and 94)

Monoamine Oxidase This enzyme almost exclusively exhibited a diffuse and evenly distributed cytoplasmic activity which was equally intense in all the ganglion cells Only a few active granules were observed in the periphery

of the neuronal cytoplasm The satellite cells were negative and the neuropil gave a very weak diffuse reaction The nuclei and nucleoli were devoid of activity (Fig 76)

The hydrazone method of Koelle and Valk gave a more intense staining than Glenner s formazan method However the distribution and nature of the reaction product were exactly the same with the two methods

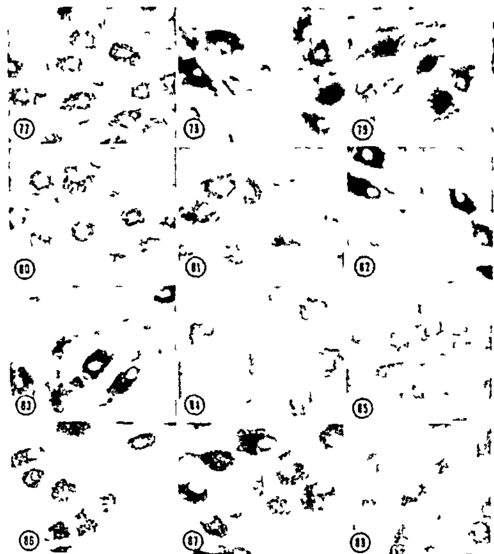
PREGANGLIONIC DENERVATION

No distinct changes were observed in the intensity or distribution of the activities of any oxidative enzymes studied in the ciliary ganglion of the rat after decentralization

POSTGANGLIONIC DENERVATION

Dehydrogenases NADH and NADPH tetrazolium reductases exhibited a pronounced and progressive increase in activity from the first postoperative day onwards The maximal reaction was reached by the 5th day (Figs 77 and 78) and a high level persisted for about a fortnight (Fig 79) On the 30th day after the operation the activity had decreased a little and reached the normal level by the 60th day (Fig 80) Both diffuse and granular activity were intensified The positive granules were chiefly concentrated in the central parts of the neuronal cytoplasm while the nuclei adopted an eccentric position The granules were larger and more intensely stained than in the control ganglion cells Some neurones gave only a weak reaction these cells were probably suffering from a deficient blood supply The activity in the satellite cells and in the neuropil did not change after axotomy DPN diaphorase activity increased much more than that of TPN diaphorase

NAD^+ linked tetrazolium reductases (only *isocitrate* and *lactate dehydrogenases* were studied after axotomy) exhibited increased activity even on the first postoperative day and the maximal activity was reached in 3 to 5 days (Figs 81 82 85 and 86) Thereafter the high level was maintained for about 2 weeks (Figs 83 and 87) After a month the activity was only a little higher than in the control and was totally normalized within two months after the operation (Figs 84 and 88) The intracellular changes in the enzyme activity were similar to those of diaphorases No significant change was found in the satellite cells or in the neuropil



The effe of postganglionic denervation on oxidative enzyme activities Fresh sections x 250

Figs 77—80 NADH tetra lium reductase NBT Incubation time 30 min *Figs 81—84* Isocitrate tetra olium reductase NBT Incubation time 30 min *Figs 85—88* Lacta e tetra olium reductase NBT Incubation time 30 min

Fig 77 Control ganglion

Fig 78 5 days after axotomy

Fig 79 10 days after axotomy

Fig 80 60 days after axotomy

Fig 81 Control ganglion

Fig 82 5 days after axotomy

Fig 83 15 days after axotomy

Fig 84 90 days after axotomy

Fig 85 Control ganglion

Fig 86 5 days after axotomy

Fig 87 15 days after axotomy

Fig 88 60 days after axotomy



The effect of postganglionic denervation on oxidative enzyme activities
Fresh sections × 250

Figs 89—93 Succinate tetrazolium reductase NBT Incubation time 30 min *Figs 94—97* Cytochrome oxidase Incubation time 60 min

Fig 89 Control ganglion — *Fig 90* 5 days after axotomy — *Fig 91* 10 days after axotomy — *Fig 92* 30 days after axotomy — *Fig 93* 60 days after axotomy — *Fig 94* Control ganglion — *Fig 95* 5 days after axotomy — *Fig 96* 10 days after axotomy — *Fig 97* 60 days after axotomy

NADP⁺ linked tetrazolium reductase glucose 6 phosphate dehydrogenase exhibited a change in activity after axotomy similar to that of other dehydrogenases However the change was not so marked as that of NAD⁺ linked dehydrogenases

α *Glycerophosphate menadione tetrazolium reductase* behaved in a similar way to the former enzyme Both particulate and diffuse staining increased The granules occupied the centre of the nerve cell beside the nucleus which adopted an eccentric position

Succinate dehydrogenase however behaved differently from all the other dehydrogenases studied. In the majority of ganglion cells a moderate increase in the activity of this enzyme was found during the first five postoperative days, while other neurones possessed normal activity (Figs. 89 and 90). Thereafter the activity began to decrease in all the neurones, reaching a minimum 10 days after axotomy (Fig. 91), and then remained at this low level for about 2 weeks. After a month the activity was only a little weaker than in the control ganglion (Fig. 92) and in 60 days it had returned to normal (Fig. 93). Both particulate and diffuse activity were affected. Only the ganglion cells showed a change in the level of this enzyme, while the interstitial tissue components retained their normal activity.

Oxidases. Cytochrome oxidase exhibited a somewhat decreased activity in the ganglion cells from the 3rd postoperative day onwards. The weakest reaction was observed on the 5th day (Figs. 94 and 95). Almost as weak an activity was also seen on the following days (Figs. 96) but it gradually began to intensify and reached its normal level by the 60th day (Fig. 97). The normally strongly positive granules were less numerous during the axon reaction, while the diffuse activity was as strong as in the control sections. The reactivity of the interstitial structures did not show any change after axotomy.

Monoamine oxidase activity exhibited behaviour similar to that of CytO after axotomy.

SYMPATHECTOMY

No clearly discernible change was found in the enzyme activities of the dehydrogenases and oxidases studied in the ciliary ganglion after extirpation of the ipsilateral superior cervical ganglion.

DISCUSSION

DEHYDROGENASES

The histochemically demonstrable activities of the tetrazolium reductases studied varied considerably in the ciliary ganglion cells of the rat The relative activities visually estimated were as follows — the most active DPN-diaphorase, isocitrate malate and lactate dehydrogenases the moderately active TPN diaphorase succinate β hydroxybutyrate glucose 6 phosphate and α glycerophosphate menadione tetrazolium reductases the weakly positive glutamate dehydrogenase the nonactive alcohol dehydrogenase Of these enzymes isocitrate succinate and malate dehydrogenases take part in Krebs cycle α glycerophosphate lactate and alcohol dehydrognases in glycolysis glucose 6 phosphate dehydrogenase in the pentose phosphate cycle β-hydroxybutyrate dehydrogenase in the metabolism of fatty acids and glutamate dehydrogenase in amino acid metabolism

The dehydrogenase activities in the ciliary ganglion of the rat mainly resembled those in the sympathetic and sensory ganglia of mammals (Potanos *et al* 1959 Klein 1960 Romanul and Cohen 1960 Samorajski 1960 Thomas and Pearse 1961 Tewari and Bourne 1963 Harkonen 1964) However in the sympathetic ganglion cells of the rat succinate and α glycerophosphate menadione tetrazolium reductases were weakly positive and glutamate dehydrogenase devoid of activity (Harkonen 1964) while in the ciliary ganglion cells these enzymes were as reactive as other dehydrogenases with the exception of glutamate dehydrogenase which was only weakly positive as observed in the present study The greatest differences compared with the motor neurones of the rat spinal cord were seen in the distribution of the succinate and α glycerophosphate menadione tetrazolium reductases These enzymes were mostly localized in the neuropil of the spinal cord (Soderholm 1965) while in the ciliary ganglion the staining of the neuronal cytoplasm was far more intense

The ciliary ganglion cells also showed individual variations in their reactions with different substrates although this variation was not great Such a variation in staining intensity may be due to differences in the phase of the metabolic cycle (Tewari and Bourne 1962a 1962b) In the present study however no correlation was found between the intense perinuclear staining and the location of the nucleolus The difference in staining between the neurones of the same ganglion may also be due to heterogeneity in the enzyme activity of mitochondria (Hess *et al* 1958 Scarpelli *et al* 1958) Szentágothai (1957) and Nandy and Bourne (1964 b) have demonstrated some oxidative enzyme activity in the synaptic structures in the ciliary ganglion and in the spinal cord No such reaction could be detected with certainty in the ciliary ganglion of the rat because it was masked by the activity in the satellite cells However the synaptic terminals contain mitochondria (De Robertis 1956) so that the activity of oxidative enzymes in synapses is understandable

The neuronal cytoplasmic reactivity was both particulate and diffuse. This was also clearly seen in the case of succinate dehydrogenase, an enzyme which has been considered to be localized exclusively in mitochondria (Scarpelli and Pearse 1958, Rodriguez De Lores Arnaiz and De Robertis 1962, Ogawa and Barrnett 1965). The diffuse activity may be due to the disrupted mitochondria, but in some cases it may also be due to microsomal activity as in the case of DPN-diaphorase (Vesco and Giuditta 196?).

Some part of the reactivity may also be due to nonspecific staining. The lipid solubility of tetrazolium salts may bring about such an effect and it is thought that all the myelin reactivity may have been due to such an artifact, "substantivity of formazans" (Pearse and Hess 1961). On the other hand tetrazolium salts may also be reduced to insoluble formazan without any enzymatic activity, "nothing dehydrogenase", probably owing to the reaction of the highly reactive sulphydryl groups in the preparation (Zimmermann and Pearse 1959, Shaw and Koen 1965). These authors stated that alcohol dehydrogenase is largely responsible for "nothing dehydrogenase" activity. Menadione and phenazine methosulphate are also able to bring about such a reaction (Hashimoto *et al.* 1963).

The glial cells also exhibited some activity, although they reacted much less intensely than the neurones. Similar activity has also been demonstrated in the sympathetic ganglion of the rat (Härkonen 1964). A low oxidative metabolism in the neuroglia has been postulated in many biochemical studies (Robins and Smith 1953, Lowry *et al.* 1954, Strominger and Lowry 1955), while Hydén and Pigon (1960) have demonstrated a high cytochrome and succinic oxidase reactivity in the oligodendroglial cells of Deiters nucleus. The degree of glial activity may have been due to the tetrazolium salts used, since it has been shown that the glial tissue reduces other tetrazolium salts better than the neuronal enzymes (Wolfgram and Rose 1959, Rosa and Tsou 1961). The glial activity observed in the present study was not due to "nothing dehydrogenase" or "substantivity of formazans", because the boiled sections and the sections incubated without substrate were totally negative. This observation is corroborated by Harkonen (1964). Therefore oxidative metabolic processes are also taking place in the satellite cells and neuroglia, which confirms the close metabolic relationship between the neurone and its glia (Hydén 1960).

All the dehydrogenases, with the exception of succinate tetrazolium reductase, exhibited increased activity in the ciliary ganglion cells after axotomy as long as decentralization had any effect. The intracellular distribution was also shifted to the central parts of the neurones. These results agree well with the earlier investigations concerning the motor neurones and the sympathetic ganglion cells (Kreutzberg 1963, Harkonen 1964, Soderholm 1965). Succinate dehydrogenase behaves in dissimilar ways in different neurones and in different species after axotomy (Howe and Mellors 1945, Kumamoto and Bourne 1963, Harkonen 1964, Soderholm 1965).

The increased reduction of tetrazolium salts after axotomy has been suggested to be due to the [illegible] number of mitochondria (Hartmann

1954 Causey and Hoffman 1955 Hudson et al 1961), to the swelling of mitochondria (Hartmann 1954 Harkonen 1964) to the dehydration which causes both these changes (Niemi et al 1960) and so on Apparently axotomy with its consequences increases the permeability of the mitochondrial membrane to the substrate and/or tetrazolium salt the result being the histochemically intensified reaction The number of formazan deposits is greater in the swollen mitochondria (Niemi et al 1960)

The increase in the activities of many dehydrogenases is attributed to the enhanced metabolism and synthetic processes in the axotomized ganglion cells as demonstrated by biochemical studies (Brattgård et al 1957)

OXIDASES

Cytochrome oxidase which is widely distributed in the tissues concerned with aerobic metabolism (Roizin 1955) exhibited high activity in the ciliary ganglion cells of the rat The neuroglia gave a stronger reaction than with dehydrogenases Similar results have been observed in the spinal and sympathetic ganglia (Hyden et al 1958 Harkonen 1964)

Monoamine oxidase activity was also seen in the ciliary ganglion of the rat although this is believed to be a cholinergic ganglion However the ciliary ganglion cells of the cat and the rabbit exhibited MAO activity as well (Koelle and Valk 1954) The reaction product was mainly nongranular in the present study although MAO is considered to be a mitochondrial enzyme (Blaschko et al 1957 Weiner 1960) Therefore the validity of the reaction was suspected The control sections however were totally negative thus indicating a true positive reaction and this as supported by the changes observed after axotomy In adrenergic ganglion cells the MAO activity is also partly diffuse and partly granular (Koelle and Valk 1954 Harkonen 1964)

The presence of MAO in a cholinergic ganglion such as the ciliary does not necessarily mean that it is of significance in the oxidation of the adrenergic transmitter substance but MAO may also be involved in the metabolism of other amines (Koelle and Valk 1954)

While decentralization had no effect on the activities of the oxidases in the ciliary ganglion of the rat axotomy caused a definite decrease in both CytO and MAO activity Similar results have been observed in the sympathetic ganglion (Harkonen 1964) and in the spinal ganglion (Howe and Mellors 1945) The diminution of enzyme activity may indicate a gradual adaptation of the neurones to the decrease in the total amount of axoplasm as suggested by Howe and Flexner (1947)

The neuronal cytoplasmic reactivity was both particulate and diffuse This was also clearly seen in the case of succinate dehydrogenase an enzyme which has been considered to be localized exclusively in mitochondria Scarpelli and Pearse 1958 Rodriguez De Lores Arnaiz and De Robertis 1962 Ogawa and Barrnett 1965) The diffuse activity may be due to the disrupted mitochondria but in some cases it may also be due to microsomal activity as in the case of DPN-diaphorase (Vesco and Giuditta 1966)

Some part of the reactivity may also be due to nonspecific staining The lipid solubility of tetrazolium salts may bring about such an effect and it is thought that all the myelin reactivity may have been due to such an artifact substantivity of formazans (Pearse and Hess 1961) On the other hand tetrazolium salts may also be reduced to insoluble formazan without any enzymatic activity, 'nothing dehydrogenase probably owing to the reaction of the highly reactive sulphydryl groups in the preparation (Zimmermann and Pearse 1959 Shaw and Koen 1965) These authors stated that alcohol dehydrogenase is largely responsible for nothing dehydrogenase activity Menadione and phenazine methosulphate are also able to bring about such a reaction (Hashimoto *et al* 1963)

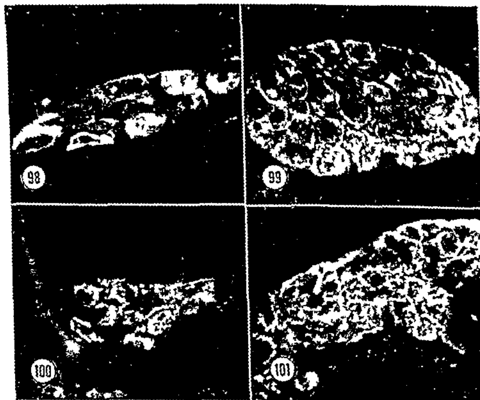
The glial cells also exhibited some activity although they reacted much less intensely than the neurones Similar activity has also been demonstrated in the sympathetic ganglion of the rat (Härkonen 1964) A low oxidative metabolism in the neuroglia has been postulated in many biochemical studies (Robins and Smith 1953 Lowry *et al* 1954 Strominger and Lowry 1955) while Hydén and Pigon (1960) have demonstrated a high cytochrome and succinic oxidase reactivity in the oligodendroglial cells of Deiters nucleus The degree of glial activity may have been due to the tetrazolium salts used since it has been shown that the glial tissue reduces other tetrazolium salts better than the neuronal enzymes (Wolfgram and Rose 1959 Rosa and Tsou 1961) The glial activity observed in the present study was not due to nothing dehydrogenase or substantivity of formazans because the boiled sections and the sections incubated without substrate were totally negative This observation is corroborated by Härkonen (1964) Therefore oxidative metabolic processes are also taking place in the satellite cells and neuroglia which confirms the close metabolic relationship between the neurone and its glia (Hydén 1960)

All the dehydrogenases with the exception of succinate tetrazolium reductase exhibited increased activity in the ciliary ganglion cells after axotomy as long as decentralization had any effect The intracellular distribution was also shifted to the central parts of the neurones These results agree well with the earlier investigations concerning the motor neurones and the sympathetic ganglion cells (Kreutzberg 1963 Harkonen 1964 Soderholm 1965) Succinate dehydrogenase behaves in dissimilar ways in different neurones and in different species after axotomy (Howe and Mellors 1945 Kunimoto and Bourne 1963 Harkonen 1964 Soderholm 1965)

The increased reduction of tetrazolium salts after axotomy has been suggested to be due to the increased number of mitochondria (Hartmann

RESULTS

A green fluorescent zone was seen to surround nearly all the ciliary ganglion cell bodies The fluorescence was of moderate intensity but very short lived In the ganglion cell cytoplasm some yellow fluorescent granules could be seen and only a few perikarya exhibited the green diffuse fluorescence typical of catecholamines The granular yellow fluorescence was seen in fresh sections even without treatment with formaldehyde vapour so that it was probably due to prelipofuscin or other autofluorescent granules The nuclei were always non fluorescent Some nerve fibres also exhibited the green fluorescence (Figs 98 and 99)



Figs 98—99 Formaldehyde induced fluorescence Normal ganglion Freeze-dried preparations × 280

Figs 100—101 Formaldehyde induced fluorescence Animal treated with nialamide (25 mg/kg intraperitoneally) 8 hours and noradrenaline (1 5 mg/kg intraperitoneally) 2 hours before death Freeze-dried preparations x 280

A number of rats were injected intraperitoneally with nialamide (N isonicotinoyl N /β [N benzyl-carboxyamido] ethyl/hydrazine) 250 mg/kg in a single dose and 5 15 hours later with noradrenaline 1 5 mg/kg in a single dose The animals were killed 2 hours after the injection of noradrenaline

As a result of this procedure the formaldehyde induced green fluorescence was much more brilliant and also more permanent Its localization however did not change but the varicose terminals were more clearly seen (Figs 100 and 101)

The control ganglia, taken from animals whose catecholamine stores had been depleted with reserpine (5 mg/kg daily three doses) did not exhibit any specific fluorescence

DISCUSSION

Since the zonal fluorescence was green and totally absent from the control ganglia and since it developed rapidly it seems probable that it was due to noradrenaline (NA) (Falck *et al* 1962 Falck and Owman 1965) Some pharmacological data indeed suggest the possible presence of adrenergic synapses in the ciliary ganglia of some mammals (Whitteridge 1937 Tum Suden *et al* 1951 1952 Perry and Talesnik 1953 Perry 1957 Martin and Pilar 1963) It has been reported that the neurones of the Edinger Westphal nucleus of the rat contain noradrenaline (Dahlstrom and Fuxe 1965)

The distribution of the fluorescent material around the nerve cell bodies coincided with the synaptic terminals It is interesting that in the ciliary ganglia of the cat such structures are very few (Hamberger *et al* 1965) whereas in the rat practically all the ganglion cells were found in the present study to be surrounded by a fluorescent ring of this kind However it was difficult to determine whether the catecholamines were located in the pre or postganglionic synaptic structures Some evidence however, suggests the former site With the electron microscope dense-core vesicles can be seen in the preganglionic synaptic terminals of the ciliary ganglion of the rat (to be published) These vesicles are presumed to store amines (De Robertis and Pellegrino De Iraldi 1961, Pellegrino De Iraldi *et al* 1963 Bondareff 1965 Eränko 1967) Furthermore the perikarya of the ciliary ganglion cells did not usually exhibit any specific fluorescence as would be the case if the catecholamines were localized in the postganglionic synaptic structures as they are in the sympathetic ganglia (Eranko and Harkonen 1963 1965)

From the fact that the preganglionic synaptic terminals also contain AChE which disappears after decentralization (see Chapter I) it can be concluded that there are terminals containing both AChE and NA in the ciliary ganglion of the rat and possibly such terminals are also present in the rat iris in which some nerve fibres contain both AChE and NA (Eranko and Raisänen

1965 Ehinger and Falck 1965 Eranko 1967) The presence and function of MAO in the ciliary ganglion cells thus becomes comprehensible (Kopin 1964)

The significance of the adrenergic synapses in the parasympathetic ganglia is unknown It has been suggested that adrenaline and NA may inhibit ganglionic transmission and that ACh is the excitatory substance (Hamberger *et al* 1965)

SUMMARY

The present histochemical investigation deals with the normal activity and distribution of carboxylic esterases phosphatases oxidative enzymes and catecholamines in the ciliary ganglion of the rat and the effect of pre and postganglionic nerve division The effect of extirpation of the superior cervical ganglion on the activities of hydrolytic and oxidative enzymes was also studied

Carboxylic Esterases (Chapter I) The carboxylic esterases were demonstrated in fresh postfixed and fixed sections using acetylthiocholine iodide butyrylthiocholine iodide α naphthyl acetate α naphthyl butyrate 4-chloro 5 bromoindoxyl acetate and naphthol AS D acetate as substrates With the aid of 284 C 51 *iso* OMPA eserine and E 600 as selective inhibitors the esterases were divided into four groups acetylcholinesterase (AChE), non specific cholinesterase (ns ChE) E 600 sensitive non specific esterase (E s ns E) and E 600 resistant non specific esterase (E r ns E)

AChE was demonstrable in all the ganglion cells the intensity of the reaction varying from weak to strong The synapses and postganglionic nerve fibres were strongly positive while only a few if any of the preganglionic nerve fibres gave a reaction Preganglionic denervation abolished the AChE activity of the preganglionic nerve fibres and the preganglionic synaptic terminals Postganglionic denervation resulted within three days in the total disappearence of AChE from the majority of the ganglion cells including both pre and postganglionic synaptic structures and nerve fibres The AChE reactivity had to some extent reappeared by the 30th day after the operation and was totally normalized by the 60th day

The ns ChE activity was strong in all the nerve fibres in the satellite and Schwann cells and in the capillaries Some ganglion cells also exhibited an intense activity After decentralization the interstitial ns ChE activity was much decreased while axotomy caused a still greater decrease in the interstitial ns ChE activity In the latter case the perikarya of the active neurones lost their activity The activities were normalized in 60 days

E s ns E activity was seen in all the ganglion cells In fresh sections the activity was intense indicating the desmo-nature of this enzyme whilst its formalin sensitivity was shown by the fact that formalin fixation and even postfixation almost totally abolished its activity Preganglionic nerve division had no effect on the E s ns E activity Following postganglionic nerve division E s ns E activity was greatly diminished after three days but was fully restored two months after the operation

E r ns E was only demonstrable in fixed sections indicating the lyo-nature of the enzyme The activity was mainly localized in cytoplasmic granules in all the neurones After decentralization and axotomy E r ns E behaved in a similar way to E s ns E respectively

Phosphatases (Chapter II) The distribution of acid phosphatase (AcPase) differed considerably in fresh and fixed sections In the former the reaction product was diffuse in the ganglion cell cytoplasm and the interstitial tissue also showed some activity in the latter the activity was mainly confined to cytoplasmic granules of the ganglion cells while the interstitium was inactive Preganglionic denervation had no effect on the AcPase activity in the ganglion After postganglionic denervation AcPase was much increased for about 30 days The normal level was reached in two months

Alkaline phosphatase (AlPase) was mainly localized in the capillaries Neither pre nor postganglionic nerve division had any effect on the activity of this enzyme

Adenosine triphosphatase (ATPase) exhibited intense activity in the capillaries and nerve fibres The satellite cells and Schwann cell and possibly also the cell membrane of the neurones showed activity After decentralization the activity was not appreciably changed while after axotomy the interstitial activity except that of the capillaries was greatly decreased in a few days Normal values were reached in 60 days The activity of the ganglion cells did not show any change after axotomy

Oxidative Enzymes (Chapter III) The histochemically demonstrated oxidative enzymes (NADH NADPH succinate β hydroxybutyrate isocitrate malate lactate α glycerophosphate menadione glutamate and glucose 6 phosphate tetrazolium reductases cytochrome oxidase and mono amine oxidase) exhibited more intense activity in the cytoplasm of the ganglion cells than in the surrounding satellite cells while the other interstitial structures showed a still weaker activity than the latter The distribution of the cytoplasmic activity was partly granular and partly diffuse Alcohol dehydrogenase was inactive in the whole ganglion

Preganglionic nerve division had no effect on the activities of the oxidative enzymes in the whole ganglion After postganglionic nerve division the activities of these dehydrogenases increased returning to normal again about 60 days after the operation After the initial increase the activity of succinate dehydrogenase began to decrease on about the 10th day after axotomy Normalization was complete in 60 days Cytochrome oxidase and monoamine oxidase activities decreased after axotomy the changes being reversible as usual

Catecholamines (Chapter IV) A weak to moderate green fluorescence localized to the synaptic terminals was seen to surround nearly all the ganglion cell bodies after treatment of the freeze dried ganglia with formal dehyde vapour The fluorescence was intensified after successive injections of monoamine oxidase inhibitor (nialamide) and noradrenaline

The extirpation of the superior cervical ganglion had no effect on any enzyme activities of the ipsilateral ciliary ganglion of the rat

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FROM THE DEPARTMENT OF PHARMACOLOGY UNIVERSITY OF UPPSALA SWEDEN

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GENERAL INTRODUCTION

Electromyography (EMG) was introduced for clinical use by Piper in 1912 He used surface electrodes A great improvement on the technique was made by Adrian & Bronk in 1929 who introduced the concentric needle electrode The method has since been developed into a useful tool for the diagnosis and study of muscular diseases

In ordinary electromyography the summated activity from a great number of muscle fibers is recorded Fibers innervated from the same motorneuron and therefore activated simultaneously, generate the motor unit potential The EMG gives information about the total electrical activity within a certain area in the vicinity of the electrode The properties of the single muscle fiber are not investigated with this method

Although the examination of single muscle fibers has long been desired there have been considerable difficulties in doing this as a clinical routine test Glass capillary electrodes for measurements of the resting potential have been used by Johns (1960) Norris (1962) Creutzfeldt (1962) and Riecker Bolte & von Bubnoff (1963) Beranek (1961) van der Most van Spijk den Hertog & Lammers (1965) den Hertog (1966) and Goodgold & Eberstein (1966) recorded intracellular action potentials with a similar method

Pierce & Wagman (1964) used a tungsten microelectrode for extracellular recording of action potentials in human muscle fibers *in situ* The electrode (with a tip diameter of 1 μ) was inserted percutaneously and held by a micromanipulator

The needle Multielectrode method of extracellular recording of single muscle fibers was first reported by Ekstedt & Stalberg (1963) The method was described in detail by Ekstedt (1964) The method is based on the use of a needle Multielectrode and is simple and fast During a one hour experimental period action potentials from 30 different fibers could be recorded However, the knowledge of the volume conduction of the action currents of a muscle fiber is at present not such as to permit any conclusions concerning the events of the muscle fiber membrane itself to be drawn from the shape of the extracellularly recorded action potentials

The needle Multielectrode technique also permits recording at two sites along the fiber and makes possible the calculation of the propagation velocity of the action potential along the muscle fiber in situ which has not previously been studied

The aim of the present paper is to develop a method for the study of propagation velocity in the normal muscle The propagation velocity has also been studied in different muscles in muscular disorders and during prolonged activity ischemia and changed intramuscular temperature

The investigations showed that the propagation velocity was subject to continuous changes during the experiment related to activity This made it difficult to use any instant numerical value of the propagation velocity as a parameter to characterize the fiber However when recording for longer periods (more than 5 minutes) the *change* in propagation velocity might give some information It was also found that there was a variability in the propagation velocity at consecutive discharges This variability was related to the innervation frequency in such a way that a short time interval from the preceding impulse enhanced the velocity for the second pulse This effect was the more pronounced the shorter the intervals between the discharges The analysis of this interval dependence the *Velocity Recovery Function* has given results that seem promising with regard to the characterizing of the muscle fiber membrane properties The Velocity Recovery Function changed during prolonged work during ischemia at low temperature and in some muscular disorders preliminarily studied

The following preliminary reports of results presented in this paper have been given Ekstedt & Stålberg (1963) Stålberg & Ekstedt (1965) Stalberg Dahlbäck & Ekstedt (1966)

SURVEY OF THE LITERATURE

Measurements of propagation velocity in skeletal muscle in situ

The first investigation on propagation velocity in the skeletal muscle was performed on the human forearm with surface electrodes by Herman (1878) who found dass die muskulare Leitungsgeschwindigkeit im menschlichen Vorderarm auf keinen Fall 16 Meter ubersteigt und wahrscheinlich zwischen 10 und 13 Meter liegt Due to the inaccurate methods used this investigation has but historical interest Mainly the same method (one surface electrode below the elbow one proximal to the wrist) was used by Piper (1909) who found the impulse velocity in the forearm to be 10 m/sec The localization of the motor end plates was not taken into account

In 1939 Eccles & O Connor when studying neuromuscular transmission, recorded electrical activity from the cat soleus, anterior tibial and peroneous tertius muscles by means of two stout collars of cotton wool around the muscle The values ranged in the soleus muscle from 2 85 to 4 8 m/sec and in the peroneous tertius and anterior tibial muscles from 5 to 6 m/sec Accurate measurements of the fastest conduction velocity could not be made but was assumed to be only slightly higher

Denslow & Hasset (1943) made measurements of propagation velocity on motor unit potentials in the voluntarily activated human muscle by inserting two concentric needle electrodes in the fiber direction in different muscles The 24 values of the propagation velocity were evenly spread between 1 3 m/sec and 12 5 m/sec with mean value of 4 95 m/sec No consideration was taken of the site of the end plate in relation to the recording electrodes and therefore these results did not represent the velocity in the muscle fibers

With two pairs of two 125 μ platinum wires spaced 3–10 mm Jarcho Berman Dowben & Lilienthal (1954) measured the conduction velocity in denervated and normal muscle fibers in the anterior gracilis rat muscle In normal fibers the velocity ranged from 3 56 to 5 63 m/sec (mean 4 3 m/sec) and in denervated fibers the velocity also fell within this range but fibrillary potentials in denervated fibers had a velocity of 2 66 to 2 84 m/sec (mean 2 73 m/sec)

The first measurements of propagation velocity by means of two electrodes

within one needle was published by Meda & Ferroni (1957) In the bevelled surface at the tip of a needle (diameter of 1 45 mm) two platinum electrodes, 60 μ in diameter were mounted 1 25 mm from each other The needle was so positioned that the electrodes were grossly parallel to the fiber direction and the propagation time was measured on enlarged photos from the interval between the recorded potentials In m quadriceps femoris the mean velocity in 6 healthy subjects was found to be 4 26 m/sec SD 0 782 n=134

Buchthal, Guld & Rosenfalck (1955*a* *b*) noticed that in the measurements of Denslow & Hasset (1943) it was impossible to ascertain whether the electrodes were positioned on the same fiber They thus considered the results achieved not to reflect the true distribution of propagation velocity in the muscle To overcome these difficulties Buchthal *et al* used a method in which they stimulated a small group of fibers in the brachial biceps in man by an intramuscular needle electrode and picked up the evoked muscle potentials with 3–5 concentric electrodes at some centimeters distance from each other along the fiber direction After 1–2 hours of searching it was possible to find a position of the needles where the recorded potentials appeared and disappeared simultaneously when the stimulation strength was changed and where there was a rectilinear relationship between the arrival time of the potentials recorded from different electrodes and the electrode distance The propagation velocity in these measurements was found to be 4 02 m/sec SD 0 45 n=7 In another series of experiments Buchthal Guld & Rosenfalck (1955*b*) presented the same type of experiments with voluntarily activated muscles The electrode positions were found with electrical stimulation as described above There was a variation in velocity exceeding by far that obtained with electrical stimulation and the authors commented that "this difference must be due to spatial dispersion of the site of innervation for simultaneously activated fibers the spikes recorded at the different electrodes not necessarily arising from the same fibers of the motor unit" The velocity was found to be 4 72 m/sec SD 0 54 n=28 No difference in velocity was found between children adults and aged persons No measurements of propagation velocity during prolonged continuous activity were made

In 1961 Sano used vector recordings from two electrodes spaced up to 40 mm When a Lissajous figure appeared on the oscilloscope it could be concluded that the electrodes recorded activity from fibers belonging to the same motor unit but no attempt appears to have been made to ascertain that the recordings were made from a single fiber Time was measured between the negative peaks of the potentials and the velocity was calculated to be 8 3 7 1 5 0 m/sec respectively (mean value 6 8 m/sec) in different paretic muscles

Even if there are reports in the literature on measurements of propagation velocity in human muscle only a few of these might represent measurements from single fibers

Propagation velocity and fiber diameter

Recent reviews of electrical properties in excitable tissue is given by Ussing (1959) and Noble (1966) Different models used for calculating the propagation velocity were discussed by Håkansson (1956)

Since the prediction of Gasser & Erlanger (1927) that there was a proportionality between the conduction velocity and fiber diameter in myelinated nerve many investigations have shown this to be true both for the myelinated and non myelinated nerve fibers The results are however, very conflicting

Since the velocity in the *myelinated nerve fiber* might be influenced by the myelin sheath and the inter nodal distance the relationship between velocity and fiber diameter is not analogous to that in the muscle fiber

In *non myelinated nerve* (giant axon of the squid) Pumphrey & Young (1938) correlated the largest diameter in the fiber bundle and the shortest propagation time in that bundle and found the velocity to vary approximately with the square root of the fiber diameter Offner Weinberg & Young (1940) obtained the same results in their mathematical analysis while Gasser (1950) and Hodes (1953) found a linear proportionality in the non myelinated nerve of the cat

Katz (1948) supposed the correlation to be essentially the same in *muscle fibers* as in non myelinated nerve fibers Håkansson (1954 1956) made the first systematic investigation of the relationship between propagation velocity and diameter of the isolated muscle fiber Because the fibers of the semitendinosus muscle in the frog have an elliptic cross section the size of the fibers were characterized by their circumferences It was found that the conduction velocity was directly proportional to the fiber circumference

For the human brachial biceps muscle *in situ* Buchthal Guld & Rosenfalck (1955*b*) found a narrow range of velocities in different muscle fibers as compared to the three fold variation in fiber diameter They considered the inactive fibers as insulators and the extracellular space smaller than had earlier been reported (6 % instead of 13 %) which should give a high external resistance masking the influence of fiber diameter

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METHODS

Recording

The electrode probes

Probes for measurement of the propagation velocity

The method was based on the use of Multielectrode probes of the same type as described by Ekstedt in 1964

Fourteen platinum wires individually isolated were introduced in an ordinary injection cannula Two millimeters from the point opposite to the bevel surface they were bent up through an opening 0 5–1 mm in the cannula The wires were arranged in a certain geometrical pattern and fixed in epoxy resin (Araldite® Ciba) The needle and isolator were ground flat The diameters of the needle were 0 6 and 0 5 mm Each wire was connected to its pin in a multipolar plug As seen in Fig 1 there were thirteen electrodes in one row and one single electrode 240 μ from the center of the row

The distance between electrode centers was about 30 μ and each electrode 25 μ in diameter

This Multielectrode with small interelectrode distances was the one most suitable for studies of propagation velocity In addition other types of multielectrode probes were used for example the *"3+11"* and *"3×4"* Multielectrodes (Fig 2) These were also used by Ekstedt (1964)

The reference electrode

In most of the experiments a concentric needle electrode subcutaneously inserted was used as reference electrode Before recording it was controlled that no electrical activity was picked up from this electrode Sometimes one of the outer electrodes in the multielectrode probe was used as reference for example when studying the cross talk (page 26)

Electrical properties of the recording equipment

A detailed description of the recording system and the method used for testing the properties is given by Ekstedt (1964) Here only some of the most important characteristics will be given

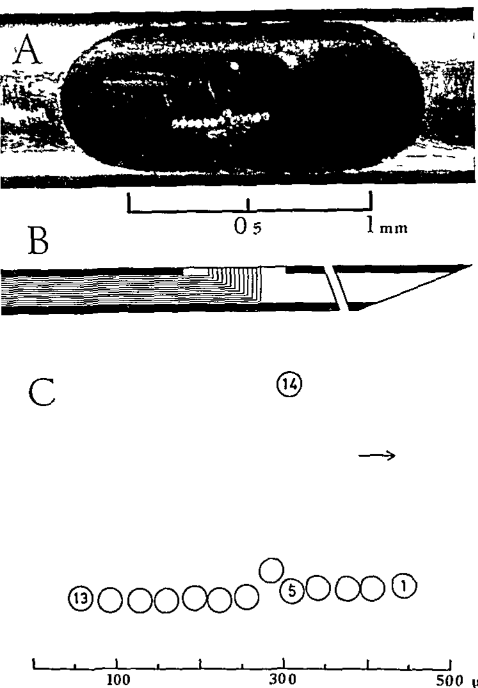


FIG 1 *The 1+13 Multielectrode* *A* Microphotograph of the electrode array *B* Schematic drawing of a longitudinal section of the needle showing the platinum wires embedded in epoxy resin and ending in the side of and flush with the needle *C* The electrode array redrawn from a microphotograph

A

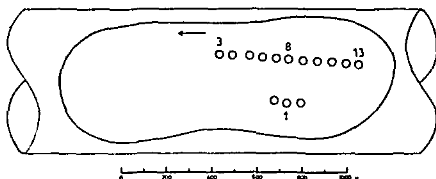




FIG 2 *The electrode array* of *A* The *3+11* Multielectrode and *B* The *3×4* Multi electrode The arrows indicating the points of the needles

Electrical properties of the amplifiers without the electrode

The input impedance was 250 Mohm and the input capacitance 3–5 $\mu\mu$F

The frequency response curve was flat between 1 and 20 000 Hz and reduced 3 dB at 0 5 and 40 000 Hz Phase distortion and nonlinear distortion was negligible

Common mode rejection was more than 700 times for any recording condition

Electrical properties of the amplifiers and the electrode connected

The frequency response curve was flat between 2 and 20 000 Hz and decreased 3 dB at 1 and 35 000 Hz

Common mode rejection was more than 300 and usually 500 times

The 10–90 % rise time for the whole system was 12 μsec

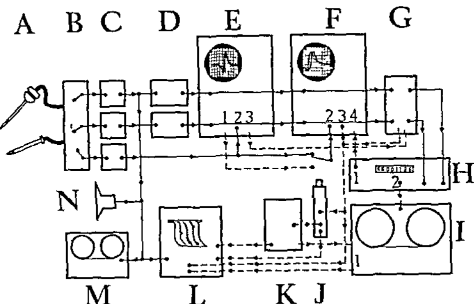


FIG 3 *The experimental set up* *A* The Multielectrode and the subcutaneous reference electrode *B* Input cathode follower and electrode selector Each electrode in the Multielectrode is connected to its own cathode follower and its connecting wire separately cathodally screened *C* The amplifiers with attenuators *D* The Delay Units in which the action potentials can be delayed up to 500 μsec *E* Dual beam oscillo scop (E1=sweep sawtooth signal output E2=External trigger input E3=Gate signal output The gate signal is simultaneous with the oscilloscope sweep) *F* Dual beam oscilloscope which is photographed (F2=External trigger input F3=Gate signal output F4=input for intensity modulation of the oscilloscope beam) *G* The Gate Unit by which the action potentials are prevented to reach the Time Interval Counter except during the passage of a sweep on oscilloscope E or F *H* The Time Interval Counter (H1=output for marking pulses that gives brightened spots on the action potential on oscilloscope F coincident with the counter start and stop H2=the binary coded decimal output to the Magnetic Tape Unit) *I* The Magnetic Tape Unit (I1=event markings to the ink jet recorder) *J* Oscilloscope camera (governed by F) *K* Time marker giving synchronous signals to camera Magnetic Tape Unit and ink jet recorder *L* Ink jet recorder for action potentials and event markings *M* Tap recorder for study of the innervation frequency *N* Loudspeaker with its amplifier

The Delay Unit

In order to be able to see the initial part of an action potential ahead of the triggering point a special device was constructed By means of this the action potential before it was displayed on the oscilloscope screen, could be delayed in relation to the original signal which triggered the sweep This Delay Unit (D in Fig 3) was built up around identical electromagnetic delay

lines in which the signal could be delayed from 0 to 500 μsec in 10 μsec steps (Czekajewski Ekstedt & Stålberg to be published) The rise time 10–90 % was 3 % of the actual delay time i e 15 μsec for 500 μsec delay High frequency response gradually decreased from 40,000 to 32,000 with increasing delay time from 0 to 500 μsec There was no phase distortion or nonlinearity within the passband The Delay Unit had two channels that were identical in every respect

Action potential display

The potentials were displayed on two dual beam oscilloscopes (Tektronix type 502 and 502A) with P11 phosphor The oscilloscopes were triggered by the potentials themselves e g by the initial positive phase This part of the potential was rather slow and therefore interference from distant active fibers sometimes gave fluctuations of the trigger point on the potential from discharge to discharge and an unstable oscilloscopic picture To get it more steady the triggering was sometimes made by the rising phase of the potential which was 4 times faster than the initial positive phase and therefore the trigger point was less sensitive to background activity The optimal stability of the oscilloscope display was attained when the base line intersection of the potential was used for triggering

The triggering technique normally used means that only the largest potentials can be dissected free of a number of nearly simultaneously appearing signals The problem of triggering action potentials at option even small ones was not easily solved except in one special case namely when the small potentials came in a fixed time relation to a large triggerable signal At voluntary contraction this occurs when two action potentials belonging to the same motor unit could be recorded at the same time To get only the small signal displayed the triggering was made in the following way One oscilloscope was triggered in the ordinary way by the large first action potential and with a sweep velocity, permitting both potentials to be seen The second oscilloscope was then triggered upon the horizontal deflection voltage from the first oscilloscope and thereby an optional delay of the trigger of the second oscilloscope could be obtained

One of the oscilloscopes was photographed by a 16 mm movie camera (Beaulieau R16) By a special device the camera shutter was open during the sweep after which a new frame was advanced The camera followed the oscilloscope up to a maximal frequency of 15/sec Several action potentials could also be superimposed on one frame Exposure number and time was indicated on each frame

Ink jet recorder

For simplifying the analysis of the experiments a three channel ink jet recorder (modified Mingograph type 24 B) was used On this the total activity from the electrode was recorded as well as time markings and event markings for oscilloscope sweeps for recordings on the digital tape and for moments of photographing with the oscilloscope camera

Measurements of propagation time

During the development of the method the propagation time i e the time for an action potential to travel the distance between two electrodes, was determined by measuring the distance between the base line intersection points of the two action potentials on an enlarged photograph

Later the method of sweep wobbling was developed to increase the accuracy of the measurements and to facilitate the control of the needle position The horizontal deflection voltage of the oscilloscope was modulated

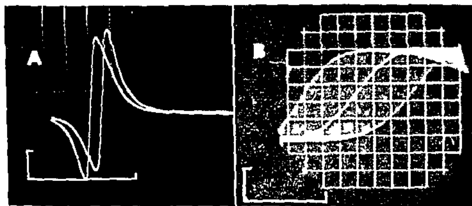


FIG 4 *Action potentials from electrodes 1 and 8 in the 3+11 Multielectrode A* Calibration 2 mV and 500 μsec The propagation time is measured between the baseline intersection points of the two action potentials Upward deflection means negative voltage Note that the two action potentials are of somewhat different form the negative peak of the first potential and the positive peak of the second potential are slightly rounded due to cross talk between the electrodes (cf page 26) The total amplitude is the same *B* Calibration 2 mV 100 μsec The *sweep wobbling* method of measuring the propagation velocity used at an early stage of the investigation (in Chapter 9) The sweep voltage is modulated by a 1 Mc sine wave which makes the two action potentials appear as broad bands The voltage of the 1 Mc signal that is needed to make the two bands just touch each other in the rising phases of the action potentials is proportional to the propagation time

by a 1 Mc AC signal The spot then wobbled to and fro one million times a second and the action potentials appeared as broad bands (Fig 4) the width of which was proportional to the voltage of the modulating signal The wobbler voltage necessary to make the two bands just touch each other in the region of the steep rising phases was a measure of the time difference between the action potentials By means of known time intervals a calibration curve was made for the conversion of potentiometer values to actual time

The wobbler voltage was adjusted with a 10 turn potentiometer and the readings were made from its scale and were dictated on a tape recorder and usually also registered on a potentiometer recorder (Varian G 10) The time intervals for 5–10 discharges were constant within 5 μsec With the use of an oscilloscope sweep of 20 usec/cm the maximum error of the method was ± 5 μsec

In the last 300 experiments time intervals were measured with a Time Interval Counter consisting of an Electronic Counter (Hewlett Packard 5245L) with Time Interval plug in unit (Hewlett Packard 5262A) The maximal error was ± 0.1 μsec ("last digit error") The drift was less than three parts in 10' per day It was important to start and stop the counter from corresponding points on the action potentials so chosen that the time interval should be insensitive to small variations in the potential amplitude The base line intersection was the most stable and reliable point for this purpose However noise and action potentials from other motor units also passed the base line and triggered the counter but this was prevented by a special gating device by which only those signals that were displayed on one of the oscilloscopes were permitted to reach the counter (H in Fig 3) The moments for start and stop were indicated by brights spots on the action potentials on the oscilloscope

The digital read out tubes of the counter were photographed by a camera (Bealieau R 16) which followed the counter to a maximal frequency of 15/sec The data from the film was punched on cards for calculations on a digital computer

In the last 100 experiments the time interval readings from the counter were directly recorded in IBM computer compatible code on magnetic tape (Magnetic Tape Unit model DY 2546 Dymec) Besides the propagation time the actual time from the beginning of the experiments in milliseconds was recorded for each impulse as well as the interelectrode distance used and the markings for different events during the experiments The maximal average recording rate was 19 data per second asynchronously However by means of a memory in which the information was stored before it was written on the tape the shortest interval between occassional measurements was 2 msec The tape was directly used as an input medium to the computer

Measurements of interspike interval

The time interval between the consecutive discharges were originally in directly determined from the recordings on the ink jet recorder with a paper speed of 100 mm/sec with an approximate accuracy of 5 msec Later the measurements were made in the following way impulses from the oscilloscope ("the gate pulses"), simultaneous with the sweeps that were triggered by the potentials were recorded on a tape recorder (Ampex type 122) with a tape speed of 19 cm/sec These pulses were then played back into the Time Interval Counter and the distance between each measured The maximal error was 0 1 msec When using the digital Magnetic Tape Unit the time intervals for each impulse were automatically measured by the Time Interval Counter and recorded on the magnetic tape The maximal error was ± 1 msec ("last digit error")

Stimulation

For electrical stimulation a double pulse generator was used with variable pulse width frequency voltage and intervals between pulse pairs A doubly shielded output transformer was used where the secondary shield was isolated from ground and connected to the shaft of the stimulation needle (Buchthal Guld & Rosenfalck, 1955*a*)

A bipolar stimulation needle (DISA 13K14) was used In order to simulate a physiological uneven innervation rhythm the stimulator could be triggered by tape recorded pulses i e the gate pulses from an oscilloscope triggered by voluntary action potentials

Temperature measurements

In most of the experiments temperature was measured with a thermoprobe consisting of a copper-constantan thermocouple close to the tip within a 0 6 mm injection cannula

The reference junction was kept in an oil bath of temperature 35 0°C ± 0 1°C The thermal EMF was measured on a DC microvoltmeter (Philips GM 6020) The SD of single readings was about 0 1°C The systematic error in temperature measurements due to thermal conduction in the thermoneedle and the recording Multielectrode (Buchthal Hønke & Lindhard 1944) is not known As the two needles are of the same material and diameter and with the same length within the muscle the systematic change of temperature around the two needles should be of the same order of magnitude The

by a 1 Mc AC signal The spot then wobbled to and fro one million times a second and the action potentials appeared as broad bands (Fig 4) the width of which was proportional to the voltage of the modulating signal The wobbler voltage necessary to make the two bands just touch each other in the region of the steep rising phases was a measure of the time difference between the action potentials By means of known time intervals a calibration curve was made for the conversion of potentiometer values to actual time

The wobbler voltage was adjusted with a 10 turn potentiometer and the readings were made from its scale and were dictated on a tape recorder and usually also registered on a potentiometer recorder (Varian G 10) The time intervals for 5–10 discharges were constant within 5 μsec With the use of an oscilloscope sweep of 20 usec/cm the maximum error of the method was ± 5 μsec

In the last 300 experiments time intervals were measured with a Time Interval Counter consisting of an Electronic Counter (Hewlett Packard 5245L) with Time Interval plug in unit (Hewlett Packard 5262A) The maximal error was ± 0.1 μsec ("last digit error") The drift was less than three parts in 10^9 per day It was important to start and stop the counter from corresponding points on the action potentials so chosen that the time interval should be insensitive to small variations in the potential amplitude The base line intersection was the most stable and reliable point for this purpose However noise and action potentials from other motor units also passed the base line and triggered the counter but this was prevented by a special gating device by which only those signals that were displayed on one of the oscilloscopes were permitted to reach the counter (H in Fig 3) The moments for start and stop were indicated by brights spots on the action potentials on the oscilloscope

The digital read out tubes of the counter were photographed by a camera (Bealieau R 16) which followed the counter to a maximal frequency of 15/sec The data from the film was punched on cards for calculations on a digital computer

In the last 100 experiments the time interval readings from the counter were directly recorded, in IBM computer compatible code on magnetic tape (Magnetic Tape Unit model DY 2546, Dymec) Besides the propagation time the actual time from the beginning of the experiments in milliseconds was recorded for each impulse as well as the interelectrode distance used and the markings for different events during the experiments The maximal average recording rate was 19 data per second asynchronously However by means of a memory in which the information was stored before it was written on the tape the shortest interval between occassional measurements was 2 msec The tape was directly used as an input medium to the computer

The subjects were ordered to keep up a slight constant contraction Single muscle fiber potentials were usually easy to find and within 30–60 seconds from the insertion of the needle the recordings could begin

During the experiment continuous small corrections of the needle position had to be made to keep the correct position Recording of data was made only when optimal conditions were present Ten to twenty potentials every 10 seconds were usually recorded but in the experiments where the digital tape recorder was used thousands of consecutive action potentials were often recorded

Recording of voltage decrement curves

The decrease in action potential amplitude at increasing distance between the muscle fiber and the recording electrode here called the voltage decrement curves were usually obtained in the following way the needle was positioned and the electrodes connected in the same way as described for recording the propagation velocity The action potentials from electrode 14 were kept

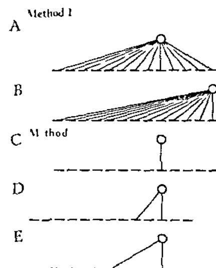


FIG 5 The two methods used to record action potentials at different distances from the same fiber (*voltage decrement curves*) The *1+13* Multielectrode is used *Method 1* The Multielectrode is kept in a fixed position in relation to the fiber and recording is made from all the electrodes one by one In A the fiber overlies electrode 5 in B the fiber overlies one of the outer electrodes (number 1) *Method 2* Recording is always made from electrode number 5 and the Multielectrode is for each recording moved so that the different electrodes one by one underlie the fiber In C the fiber is positioned over electrode 5 In D and E the fiber is positioned over electrode 3 and 1 respectively and recording is made from electrode 5

constant on one channel to provide a check of the needle position while potentials from each of the other electrodes were photographed (Figs 5A and 6) In some experiments the needle was placed so that the fiber passed one of the outer electrodes (e g electrode 1) which also served as control of position The other electrodes were then connected successively In this way longer electrode fiber distances were obtained (Fig 5B and 7)

FIG 6 *Voltage decrement recordings mad according to method 1 in fig 5* Channel 1 6 superimposed action potentials from electrode 14 Channel 2 action potentials from electrodes 6–1

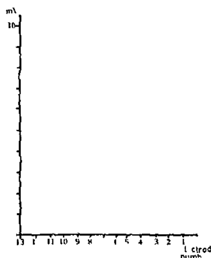
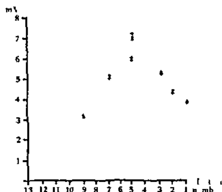


FIG 7 *Amplitude of action potentials recorded at different distances from the fiber in one experiment* Recording has been made according to B in fig 5B i e the fiber overlies electrode 1 and the recording is made from the electrodes in the long row in succession Note that the distances on the abscissa are the distances along the electrode row not the actual distances to the muscle fiber which are unknown Abscissa recording electrode The distance between electrode 13 (from which no recording was made in this experiment) and electrode 1 is 387 μ Ordinate Action potential amplitude in mV

FIG 8 *Amplitude of action potentials recorded at different distances from the fiber* (●) are the values obtained when recording is made according to A in fig 5 The fiber overlies electrode 4 and recording is made from the other electrodes in the long row The other values are obtained when recording was made according to C–E in fig 5 i e recording was made from electrode 5 (+) and 14 (▲) while the Multielectrode was moved so that the electrodes underlay the fiber one by one



Another method for obtaining the voltage decrement curves was to move the Multielectrode so that the fiber passed over each electrode surface (Fig 5C–E) Recording was made from electrode 14 and from the corresponding electrode in the long row each time the fiber passed a new electrode (Fig 8)

Analysis of data

Most of the calculations in this investigation have been made on the Control Data Corporation 3600 computer of the Uppsala Data Center The principles for the statistical analysis were taken from Snedecor (1956) (t a test of differences p 45 analysis of regression and correlation p 122 curvilinear regression p 447) For calculating the mean propagation velocity the arithmetic mean has always been used

Addresses

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CHAPTER 2

HOW TO POSITION THE MULTIELECTRODE CORRECTLY AND ESTIMATION OF ERRORS IN MEASUREMENTS

SUMMARY

The following criteria for correct position of the Multielectrode for measurements of the propagation velocity were found

1 The action potentials from the two recording electrodes had maximum amplitude and parallel rising phases at the same position of the Multielectrode during to and fro movements

2 The measurement of the time interval should be made between the zero intersection points of the action potentials in the needle position when they were of maximum amplitude

3 During these recording conditions the maximal errors in measurement of propagation velocity over the needle was +4 % to -2 %

INTRODUCTION

The Multielectrode method of determining the propagation velocity is based on measurements of small time intervals and distances and the results therefore are dependent upon the accurate positioning of the electrodes in relation to the muscle fiber

The present chapter is aimed at developing the criteria for correct needle position and to elucidate the sources of error in the method that are due to solely technical factors The possible influence of the presence of the needle electrode upon the propagation velocity itself is discussed in Chapter 4

Procedures for placing the needle in correct position

In order to be able to measure the propagation velocity of an action potential over a short distance (about 250 μ) the exact position of the electrode in relation to the fiber has to be known for calculating the actual propagation length along the fiber To get the exact propagation length the line between the two recording electrodes must be parallel with the fiber When this is the case the distance between the electrodes is the same as the propagation length

The "to-and fro" procedure

The action potentials from electrode 14 were displayed on one channel and the $1+13$ Multielectrode was moved into the muscle until a fiber action potential was obtained Then the electrodes in the long row were connected to the oscilloscope one by one, and the one giving the biggest action potential was chosen for recording and displayed on channel two

The Multielectrode was now slightly moved to and fro ($\pm 60\ \mu$) in the direction of the needle axis When the needle was out of the optimal position the amplitude was decreased but the action potentials from the two electrodes closely resembled each other in form in all needle positions When the fiber passed exactly over the connected electrodes, they registered maximal amplitude at the same position of the needle when it was moved to and fro

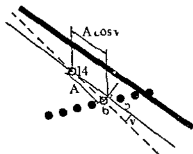


Fig 9 *Wrong angle between the muscle fiber and the electrode row* The fiber passes electrode 14 to a point midway between electrodes 5 and 6 The propagation time measured between electrodes 14 and 6 will decrease with increase of the angle v All action potentials that are recorded from a line perpendicular to the fiber must have simultaneous base line intersections (see fig 6) Therefore the length over which the propagation time is recorded must in this case be A cos v and the real distance between electrodes 6 and 14 is A If the angle v were 90° the propagation length should be zero and thus the base line intersections of the action potentials from electrodes 14 and 6 should be simultaneous

Experiments with wrongly positioned needle

Some experiments were performed to test the least error in needle position detected with the to and fro procedure

When the electrode nearest to the optimal one was connected it was seen that the recordings were of maximal amplitude at different position of the needle When the fiber was made to pass from electrode 14 to the area between two electrodes in the long row (the amplitude of the bipolarly recorded action potentials between these two electrodes was nearly zero) and the to and fro movements were made it was just possible to detect the differences in needle position for maximal amplitude of the two potentials

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When a fiber passed e g electrodes 14 and 5, the cross talk of the action potentials in electrode 1 mounted by about 4 % and the volume conducted action potential by 10 % of the maximal amplitude When switching from the subcutaneous electrode to electrode 1 as reference electrode the difference in the potentials from electrodes 14 and 5 (the fiber passing these electrodes) became less pronounced

These experiments demonstrated that the electrodes were at the same distance from the fiber when the peak to peak amplitudes of the potentials were equal even if the ratio between negative and positive phase was unequal

Experiments with rotation of the needle

Now when the appearance of the recording for optimal position was known, some experiments were made to investigate the minimal error in needle position detected by means of this criterion

The Multielectrode was rotated in both directions from the optimal position Differences in the amplitude were obvious when the needle was rotated more than 20° In this case the small potential had an amplitude of about 80 % of the large

When the needle was incorrectly placed there was also a change in the steepness of the rising phase depending on a decreased amplitude and an increased rise time on one of the action potentials For accurate study when the rising phases were non parallel certain corresponding points of the rising phases of the two potentials were superimposed by means of the Delay Unit When the potentials were parallel all points of their rising phases coincided The rising phases were seen to be non parallel when the needle was rotated about 10° and the differences in amplitude about 10 % with a sweep of 100 μsec/cm The change in propagation time could not be certainly determined in experiments but seemed not to exceed 2 %

Discussion

Cross talk was always seen to be more or less dependent on the amplitude and steepness of the rising phases of the action potentials The cross talked signal was composed of two parts one greater caused by the action potentials in the long row and one smaller caused by the action potential from electrode 14 Thus the recording from one electrode in the long row was influenced by an in phase cross talk from several electrodes and an out of phase cross talk from one electrode (number 14) The recording from electrode 14 was influenced only by out of phase cross talk from several electrodes Because of the time interval between the potentials with the steepest part of the rising

phases approximately simultaneous with the positive or negative peak of the other potential the cross talk mainly influenced the amplitude of the potentials If the electrodes had been more spaced causing a longer time interval between the recorded potentials, the cross talk would have influenced the slow positive or negative phases of the potentials (which was seen in the recordings with the *3×4* Multielectrode)

In the *1+13* Multielectrode used in the measurements of propagation velocity even the electrodes in the end of the long row 1 and 13 record d too large a volume conducted potential to be useful as reference Therefore small differences in the potential forms had to be accepted As the cross talk nearly always had its maximal influence before the positive and after the negative peak respectively the rising phases were not appreciably influenced There fore, even if one of the potentials was more distorted by cross talk than in the other and their amplitudes therefore somewhat different the rising phases should be parallel in the correct needle position

When the needle was rotated more than 10° out of correct position this was observed and corrected

Reference point for time measurements

Which part of the potential should be chosen for measurement of the time interval b tween the action potentials recorded from electrode 14 and one electrode in the long row?

When the potentials were identical in shape (except for the slight deformation caused by cross talk) and parallel in their rising phases it naturally did not matter which part of the potential was chosen for measurements as long as the measurements were made at the same level above or below the base line If the measurements were made at different levels of the two potentials quite erroneous results were of course obtained Therefore in all the experiments the measurements were made at the same level of the potentials within ±0 15 mV which is ±1 % of maximal amplitude

If however the potentials were not parallel because of small differences in the recording distances the measurements were influenced by the choice of reference point

Experiments

Some experiments were performed to evaluate the most stable and reliable reference point

When the fiber passed 14–5 and recordings were made from the electrodes

in the long row one after the other it was at once seen that they had a common base line intersection Fig 6

When time measurements were made exactly on the base line about the same propagation time was obtained in the different recordings, except for the differences arising because the electrodes did not lie exactly on a straight line When the measurements were made above and below the base line shorter or longer time intervals were recorded Fig 11

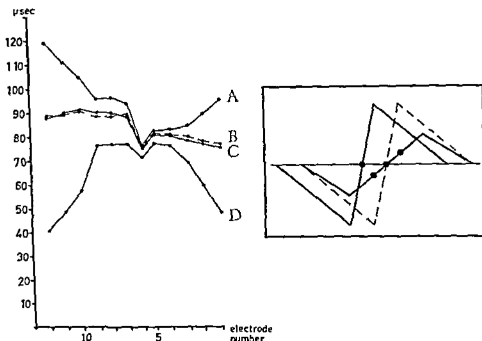


FIG 11 *Influence of the reference point on the measured value of the propagation time* The inset illustrates the principles the fiber is made to pass electrodes 14 and 5 in the *1+13* Multielectrode Measurement is first made between the base line intersections of the action potentials from these two electrodes then between electrodes 14–6 14–7 and so on The propagation times (ordinate) so obtained are plotted against the distance along the needle (abscissa) (curves B and C) The dip in the curves is caused by the fact that electrode 6 is positioned somewhat out of line The incline of the curve is caused by the fact that the long row is not strictly perpendicular to the line that joins electrodes 14 and 6 When measuring to a point 0 5 mV above the baseline of the second action potential (curve A) the time interval measured between electrodes 14 and 6 increases and this effect becomes considerably more pronounced when recordings from electrode 14 to one of the outer electrodes in the long row are made Similarly when increasing to a point 0 5 mV below the base line of the second action potential (curve D) the measured propagation time is decreased and this effect becomes more pronounced on the outer electrodes Thus the influence of incorrect position of the needle on propagation time must be least when measurement is made exactly in the base line intersection points of the two action potentials

When the measurements were made 1 mV from the base line on an action potential and picked up by an electrode nearest to the optimal one propagation time was increased or decreased by about 5 %

Discussion

According to Lorente de No (1947) and Håkansson (1957) the base line intersection point of the action potentials corresponds to that moment when the depolarisation front is projected on the recording electrode perpendicular from the fiber When therefore the action potentials were recorded at different distances from the fiber along the same line perpendicular from the fiber they had common base line intersections but different amplitudes and steepness of the rising phases What is the magnitude of error introduced into time measurements due to inaccuracy in reference point? From the experiments with different reference points for measurements it could be calculated that when the fiber passed midway between two electrodes in the long row i e the position was just as inaccurate as to go undetected by the to and fro test and one reference point was 0 15 mV above and the other 0 15 mV below the base line the error in time measurements should be less than 0 2 %

The calculation of maximal errors of propagation time measurements because of wrong needle position with the recording still fulfilling the criteria for correct position

The calculations will be based upon the fact known earlier and also confirmed in these investigations that the potential base line intersection is simultaneous for all action potentials recorded along a line perpendicular to the fiber

The experiments demonstrated that when the two basic procedures for correct positioning have been carried out the velocity could still be erroneous due to some insensitivity in these procedures

With the needle correctly positioned according to the to and fro procedure the fiber might nevertheless pass from electrode 14 to a point midway between two electrodes in the long row corresponding to a misalignment of 3 6 % The *decrease in actual propagation* length *can be trigonometrically* calculated The length will be A cos v where A is interelectrode distance used in the calculations and v the angle between supposed and actual fiber direction (Fig 9) The calculated velocity was A/t and the actual velocity A cos v/t For v = 3 6° the velocity value thus should increase by 0 2 %

The same discussion was valid for the rotation experiments When the needle was rotated 10° (which is the maximal error that passed undetected page 27) the actual distance was A cos v and the velocity should be 1 5 % too high Fig 10

Alteration of the time interval due to distortion of the potential form in the recording system

Some experiments were performed to obtain an expression of the maximal error caused by cross talk

In the *1+13* Multielectrode the outer electrodes usually registered a small volume conducted potential (5–10 % of maximal amplitude see Fig 6) and the cross talked signal Time measurements were made when the signal from electrode 1 either a) was subtracted from the preceding potential b) added to the second potential or c) these combined

This was made first when the propagation direction was 14–5 then when it was 5–14 Different results were obtained when an in phase signal or out-of phase signal was subtracted from the first or added to the second signal The cross talk was mainly "in phase" with the recording obtained from the elec trode in the row

With this "artificial cross talk" the measured time could be changed by 3 μsec shorter or longer It seems reasonable to assume that during ordinary conditions the error was less than 1 5 μsec and the variability of the error from discharge to discharge must be unimportant

Inaccuracy of the time measurement equipment

Most of the time measurements were made with the Time Interval Counter The inaccuracy in time measurements was tested by measuring the time interval between one recorded action potential and the same action potential delayed 50 μsec (with the D lay Unit, page 15) The time values obtained were 49 9 50 0 and 50 1 μsec (SD = 0 038 n = 181) The same results were obtained irrespective of whether a low high or irregular discharge frequency was used The test was also performed in the same way with sine waves with the same result

Inaccuracy of measurement of interelectrode distance

Microphotographs of the needle and an object micrometer scale (J D Moller Wedel in Holstein) were enlarged 2 000 times for measurements The

accuracy was about $\pm 2\ \mu$ corresponding to ± 4 mm on the enlarged photograph

Evaluation of the errors tested with the 3×4 Multielectrode

A single fiber potential was recorded over electrode 2 and 10 Then measurements were made 2–10, 2–6 and 6–10 The needle was then moved until the fiber passed 3–11 and new measurements were made in the same way By moving the needle along its axis twelve different interelectrode distances 50 80 93, 103, 106 144 163, 184, 194, 244, 247 and 256 μ were obtained for measurements of propagation velocity Representative results of one of the ten experiments are shown in Fig 12 The experiments were made under especially unfavourable experimental conditions because large movements of the needle were necessary The velocity decrease during these experiments seemed to be the same as in the normal experiments The standard deviation of the velocity values (from the different distances) about the sliding mean was 0 15 m/sec with a range of 0 4 m/sec In the experiment presented in Fig 12 the recordings form the electrode pair 6–10 gave a lower

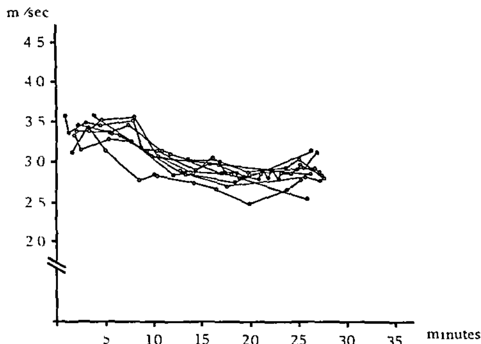


FIG 12 *Propagation velocity from one fiber recorded with the different electrode combinations in the 3×4 Multielectrode* Insertion of the Multielectrode at time zero

velocity but the measurements for the other short interelectrode distances did not seem to deviate systematically from the mean It was somewhat difficult to get acceptable recordings when the shortest distance was used (1–5) as the cross talk seemed to influence the steep rising phase

Discussion

The systematic error arising from wrongly measured electrode distance and the effect of cross talk ought to cause measurements from certain elec trode pairs to deviate significantly from the mean

As shown earlier in this chapter the cross talk had negligible influence upon the measurements when the time interval between the potentials was more than about 50 μsec (in these recordings valid for all measuring distances except for 1–5 6–10 and 4–8) but when the interval was less the cross talk signal itself decreased in amplitude The lower velocities obtained from the 6–10 distance cannot therefore reasonably be ascribed to the cross talk distortion but rather to unknown factors in this experiment In other experiments the velocity measured over 6–10 did not differ from that over other electrode pairs

The frequent repositions of the Multielectrode did not seem to influence the slope of the velocity time curve

The variations in propagation time measurements were about 10 % (SD) in most of the experiments with the *3×4* Multielectrode which is more than the errors in measurements with the *1+13* Multielectrode This was partly due to difficulties in exactly positioning the *3×4* Multielectrode because the electrodes were more spaced with the to and fro procedure it was not possible to detect small misalignments in electrode position If the needle was wrongly positioned in the plane of the electrodes and the electrode then was rotated to fulfill the amplitude and parallel criteria two misalignments were superim posed and the errors in measurements added

CONCLUSIONS

The errors in measurements are of three different types

1 Systematic errors constant through the investigation, mainly due to errors in measurements of the interelectrode distance which give a maximum error in propagation velocity of ±1 %

2 Errors due to technical factors that could change during the experiment and give slow variations of propagation velocity

a) faulty positioning of the electrode which could give about 1 5 % too short a propagation time i e 1 5 % too high a velocity value

b) wrong reference point for time measurements, giving an increase or decrease in propagation value by 0 2 %

c) error in the time interval between the potentials due to cross talk maximally ± 1 5 μsec which corresponds to ± 1 % error in the propagation value if the correct propagation time is assumed to be 75 μsec

Should these factors (2a, 2b, 2c) all tend in the same direction the maximal error in propagation velocity should be +2 7 % to -0 2 %

3 Errors in measurements of consecutive action potentials (mainly due to last digit error in the Time Interval Counter) This was maximally 0 1 μsec corresponding to 0 13 % for a propagation time of 75 μsec

Transient fluctuations in the mains voltage (stopping and starting machines in the building) could incidentally give erroneous values of the propagation time (±5–10 %) during approximately one second These errors were usually recognized but small changes might have passed undetected The fault has been corrected and was not present during the last 27 experiments

The maximal error in the measurements of propagation velocity are thus +4 % to -2 %

STIMULATION OF MUSCLE FIBERS

SUMMARY

1 For the stimulation in the human muscle in situ a bipolar stimulation needle electrode was used For stimulation of the exposed rabbit muscle two tungsten wires were also us d

2 The most common type of response obtained was a polyphasic potential Some times clean single fiber action potentials were obtain-d

3 This type of response disapp ared at curarisation of the rabbit Therefore the muscle fibers were assumed to be activated indirectly via the nerve

4 With the use of longer pulses and higher stimulation voltage coarse potentials of long duration without any spike components were obtained

5 This type of response remained unchanged during curarisation of the rabbit and was therefore regarded as caused by direct stimulation of the muscle fibers However it was not possible to limit the stimulation to one or a few muscl- fibers

INTRODUCTION

This chapter is aimed at elucidating whether it is possible to obtain a single muscle fiber activity by direct el-ctrical stimulation of the muscle *in situ*

METHODS

Human experiments

The experiments were performed on twelve subje-ts 22–35 years old and without signs of neuro muscular disease The following muscles were used the brachial biceps muscle (about 25 fibers in 5 different subjects) the extensor digitorum communis muscle (about 100 fibers in 12 different subjects)

Two electrode probes were used one Multielectrode (*1+13*) and one bipolar stimulation electrode (DISA 13K14)

The stimulating electrode was positioned distally to the end plate zone avoiding in this way coarser nerve trunks in the muscle The Multielectrode was inserted some centimeters proximally and positioned so that it recorded electrical a-tivity from the stimulated fibers The position in relation to the end plate was determined from the direction of the voluntary action potential over the probe During the period of searching for optimal recording of single fiber activity the stimulation frequency was held at 0 5–2 impulses/sec The stimulation strength was kept as low as possible slightly above that value at which some of the stimuli became ineffective

Animal experiments

Twelve experiments were performed on rabbits The animals were anesthetized by intravenous injection of sodium pentobarbital (40 mg/kg body weight) Additional doses of sodium pentobarbital were given when needed In ten experiments the skin and fascia overlying the tibialis anterior muscles in rabbits were carefully cut through and precaution was taken not to damage the superficial layer of muscle fibers The muscle surface was moistened with a 0 9 % saline and kept warm with an ordinary lamp (60 W) at a distance of 20–30 cm

The muscle was stimulated with two tungsten wires 150 μ in diameter and isolated except for the sharpened point 5–10 μ in diameter The wires were mounted in a micromanipulator and connected to the ordinary output transformer of the stimulators

As these experiments were carried out in order to investigate whether it was possible to obtain a stimulation such that single fiber activity could be recorded with a Multielectrode different stimulation techniques were tried and compared The distance between the wires was changed from 0 5 mm to 50 mm different stimulus strength and different width of the pulses 60–10 000 μsec were used In some experiments the stimulation was made superficially in others the wires were pushed into the muscle some millimeters Recordings were made with the Multielectrode either placed just on the superficial fibers or inserted into the muscle The procedure was performed under a binocular dissecting microscope with magnification 6–16 times which was also used for inspection of the stimulation result

Experiments with curare and sodium citrate In order to study the muscle response to electrical stimulation when no indirect stimulation (via the n rve) was possible two rabbits were given 3 mg d Tubocurarine intravenously (about 1 mg/kg) during continuous stimulation with an intramuscular electrode (DISA 13K14) and recording with the Multielectrode The animals were artificially ventilated via an endotracheal cannula and kept at room temperature (25–30°C) During the injection the tibialis anterior muscle was studied and when the muscle was totally paralyzed the gastrocnemius femoral quadriceps brachial biceps and triceps muscles were also examind

By means of sodium citrate it is possible to elicit electrical activity in nerve and muscle fibers because of its calciumbinding effect During the total paralysis the 0 1 ml sodium citrate was injected locally in the muscle and the Multielectrode was inserted about 5–10 mm distally to the injection area and the spontaneous activity recorded (cf Ekstedt 1964)

RESULTS

Findings common to both the human and animal experiments

Two typ s of electrically evoked responses were recorded from the electrode when it was optimally positioned (Figs 13 and 14 upper left) When the stimulating electrode was inserted distally in the muscle where no nerve fibers were supposed to be present the most usual response was a coarse action potential with a maximal amplitude of about 30 mV and with a small positive and greater negative peak This potential had no fast "spike

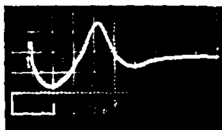


FIG 13 *Direct muscle response* obtained at electrical stimulation of the rabbit muscle during curarization Calibration 5 mV and 1000 μsec

components" and had a rise time of 0 5–1 msec and a total duration of up to 10 msec When the stimulation strength was decreased the amplitud gradually diminished to zero Despite searching for at least 20 experimental hours no recording could be obtained in which a single muscle fiber action potential could be discerned

When the stimulating electrode was placed more proximally but sometimes also with the same position of the stimulation electrode as above another type of activity was normally recorded with the use of a considerably shorter pulse duration and lower stimulation voltage There was a complex polyphasic potential form sometimes with up to 30 peaks and a total duration of up to 15 msec, depending on the distance between stimulation and recording needle When the stimulation strength was decreased the spike components disappeared one by one or a few at a time without interfering with the amplitude of the other spikes Finally a potential remained in which it was sometimes possible to obtain clean recordings of single fiber action potentials These potentials did not have a constant time relation to the other part of the complex potential, but varied about ± 10 μsec

At high stimulation strength both types of responses could sometimes be seen The latency of the polyphasic response could in different experiments vary between half and double the latency of the coarse potential

Sometimes one of the spike components in the polyphasic potential had an opposite impulse direction in relation to the others

Animal Experiments

The microscopical observation of the muscle during the stimulation with the tungsten wires disclosed that a great part of the muscle was activated even by rather weak stimulation and that small changes in position of the stimulation electrode some millimeters changed the twitch considerably

Under the influence of d-Tubocurarine the complex polyphasic response disappeared completely after an interval of about 5 minutes (Fig. 14) and in this time also the twitch disappeared When the stimulation electrode was moved in the muscle no response could be obtained with the stimulation

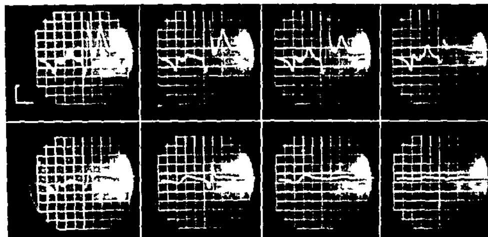


Fig 14 *Changes in the electrical response during injection of d Tubocuraine into a rabbit* The exp riments made in the tibialis anterior muscle The first re ording shown is about 3 min after injection 1 mg/kg the last recording was made 7 seconds later The components of the polyphasic response disappear and only a course response of low voltage and long duration remains Calibration 2 mV and 1000 μs c

parameters used earlier but when the pulse length was increased to 500 μsec and the stimulation strength was increased about 3 times a coarse action potential could be obtained which gradually disappeared when the stimulus strength was d creased The same results were obtained when other muscles (gastrocnemius femoral quadriceps brachial biceps and triceps muscles) were examined during curarisation

When sodium citrate was injected intramuscularly in the curarized muscle, muscle fiber action potentials could be recorded about 10 seconds after the injection During the first 30 seconds of this activity there was a high impulse frequency after which the activity either stopped or continued at a slower frequency for many minutes

DISCUSSION

d Tubocurarine has no effect on the muscle fibers but blocks the neuro muscular transmission It was also certified by means of chemical stimulation that the muscle fibers conducted action potentials normally during curari sation

The experiments with the use of d Tubocurarine have provided evidence that the complex polyphasic type of response obtained by electrical stimula tion was caused by nerve stimulation and the coarse potential type (that could only be obtained with the use of higher stimultation voltage and long r

pulse duration) was a result of direct muscular stimulation This is in close accordance with the findings of e g Krnjevic & Miledi (1958) who found that the nerve branches had a much lower threshold than the muscle fibers The finding that it was not possible to elicit single muscle fiber activity with this type of stimulation technique contrasts with the results in the human brachial biceps muscle obtained by Buchthal Guld & Rosenfalck (1955*a*) who have made a claim to have stimulated muscle fibers directly with the same type of stimulation electrode

When an intramuscular nerve twig was stimulated the whole motor unit was activated However, even then it was possible to select one of the muscle fibers for recording in the same manner as for recording during voluntary activation

These facts make it impossible to calculate the exact propagation velocity over a longer distance by means of the latency between stimulus and action potential and the distance between the stimulating and recording needles

CHAPTER 4

PROPAGATION VELOCITY DURING PROLONGED ACTIVITY

SUMMARY

1 The propagation velocity was measured with the Multielectrode technique during continous voluntary or electrical activation The brachial biceps extensor digitorum communis and frontal muscles were investigated

2 The velocity decreased during activity the decrease being most pronounced during the first 10 minutes During this time the velocity on an average decreased in the brachial biceps muscle from 3 37 to 2 92 m/sec and in the extensor digitorum muscle from 3.34 to 3 09 m/sec The difference in velocity decrease in these two muscles was not significant Activity from one fiber in the frontal muscle was recorded for more than 10 minutes The velocity decreased totally 0 01 m/sec during 10 minutes

3 In experiments with electrical stimulation it was seen that the slope of the decrease curve was dependent on the stimulation frequency Similar findings were made in the voluntarily activated muscle

4 When the needle was inserted 30–50 minutes before the activity began the same slope of the decrease curve was obtained as when the activity started at the time for insertion of the needle

5 When short periods of rest interrupted continous activity the velocity was higher after the pause This recovery was considered as a normalisation of the velocity

6 The decrease in velocity was dependent on activity but might have been more pronounced due to the presence of the Multielectrode

7 The initial velocity was assumed to reflect the propagation velocity in the muscle fiber most closely if no Multielectrode were present

INTRODUCTION

Propagation velocity decreases sometimes as much as 50 % during 30 minutes of activity Such a pronounced effect has not been reported in the literature The aim of this chapter is to study the propagation velocity decrease and some of the factors which influence it

METHODS

Propagation velocity was measured with the *1+13* Multielectrode Temperature was recorded in 10 experiments with the thermo needle kept in constant position in the muscle throughout the experiment The recordings were usually made from voluntarily activated muscle fibers The experiments were performed on 29 healthy men in the brachial biceps muscle (555 fibers) the extensor digitorum communis muscle (177 fibers) and the frontal muscle (29 fibers) Activity from

a total of 25 of these fibers has been recorded for more than 10 minutes Despite the fact that the subjects were asked to keep the innervation frequency constant there was a certain irregularity This made the interpretation of the results more difficult Therefore some experiments were performed on electrically activated fibers with constant innervation frequency In the brachial biceps muscle 114 fibers and in the extensor digitorum communis muscle 41 fibers were studied The fibers recorded were stimulated indirectly via an intramuscular nerve (Chapter 3)

The points in the figures indicate the mean propagation velocity over a 10 second period when not otherwise stated

RESULTS

A *Continuous contraction*

The activation of the muscle was begun simultaneously with the insertion of the needle and the recording started as soon as the optimal recording position was obtained Recording was however, not possible during the first 10–30 seconds after the insertion of the needle (the phenomenon of initial silence page 20)

The instantaneous repetition rate of action potentials over a period of 60 seconds was calculated The coefficients of variation ranged from 8 9 % to 21 5 % in 10 such periods

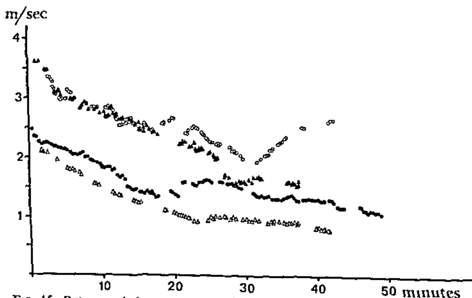


FIG 15 *Decrease of the propagation velocity during 40–50 minutes of activity in 4 fibers* Abscissa time Ordinate Propagation velocity insertion of the Multielectrode at time zero

The mean repetition rate over the first 10 seconds of every minute was calculated over a period of 30 minutes The coefficients of variation in 10 experiments ranged from 9 6 % to 22 2 % The mean frequency at the end of the 30 minutes was the same as at the beginning

In Fig 15 results are presented of propagation velocity measurements during continuous contraction Initially there was a faster decrease and after 20–30 minutes a constant level, about 50 % of the initial, was sometimes reached sometimes the velocity decreased still more In some experiments the velocity, after a normal decrease during 20–30 minutes began to increase towards initial values

The different fibers had different initial propagation velocity values and different slopes of the decrease curves (Table 1) There seemed not to be any correlation between decrease rate and initial propagation velocity Therefore the decrease in Table 1 is given in m/sec During the first 10 minutes of activity the decrease in propagation velocity appeared to be the same in the brachial biceps and the extensor digitorum communis muscles

The longest experiment with an individual fiber lasted 3 hours and 17 minutes (about 100 000 action potentials) after which time the recording was intentionally finished During this time the electrode to fiber distance did not vary more than 15 μ

Usually the experiments were stopped earlier due to one of the following reasons

a) technical imperfection so that the needle position was changed in relation to the fiber studied because of uncontrolled movements

b) The action potential being of essentially constant form during many minutes (10–20 minutes) disappeared after a gross change in the potential form during the preceding 30 seconds

c) The action potential form changed after only a few discharges to a long and low wave with a small negative phase and became impossible to record for more than 1 minute

d) The potential just disappeared without any warning

B *Influence of the discharge frequency on the propagation velocity*

To achieve a constant innervation frequency electrical stimulation was used

At the same frequency the slopes of the velocity curves for different fibers differed considerably When one and the same fiber was repeatedly stimulated with 2 and 10 pulses per second the curves obtained with each freguency were stimular but differnt for the two frequencies In Fig 16 it was shown that the propagation velocity decreased when the fiber was stimulated with 5 and 10 impulses per second When the frequency after 4–7 minutes

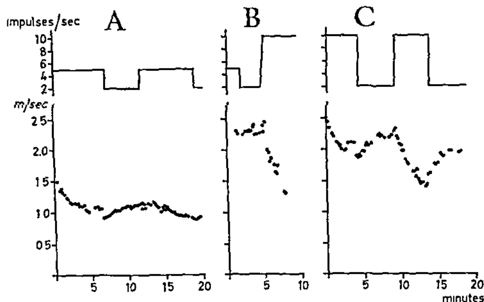


FIG 16 *Influence of innervation frequency on the propagation velocity* The muscle fiber was indirectly stimulated via its nerve and recording made with the *1+13* Multi electrode *A* Frequency 5/sec gives a decrease of the velocity Frequency 2/sec hereafter gives an increase towards the initial value *B* and *C* With the use of stimulation frequency 10 sec the decrease in velocity is greater than when using 5/sec At 2/sec the velocity increases towards the initial value again

of continous stimulation was changed to 2 impulses per second the propagation velocity increased towards the initial values

The decrease in propagation velocity when changing from a low to a higher stimulation frequency was the more pronounced the long r the stimulation proceeded

Essentially the same results were obtained with voluntary activation of the muscle Because of disturbances from other motor units recruited at stronger contraction recordings were usually not possible at innervation frequencies exceeding 15 impulses/sec Because of the inevitable irregularities in innervation rhythm at voluntary contraction the curves thus obtained have not been subject to a closer analysis

The results from these experiments showed a correlation between the innervation frequency and d crease in velocity, but it seems likely that other factors than the innevation frequency also influence the decrease in velocity

C *Effect of pauses*

When the activity was interrupted for 50–150 seconds and then resumed it was found in 30 out of 35 cases that the propagation velocity was increased by 0 05–0 4 m/sec after the pause Effects of resting periods longer than 3 minutes were not studied because of inability to keep the Multielectrode in position for such a long period without fiber activity After a pause the first registration was not made until the needle was adjusted to optimal position again After the pause the downward slope was steeper than it had been before the pause (Fig 17)

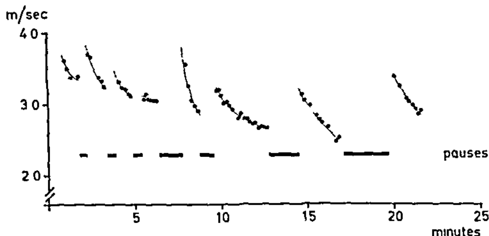
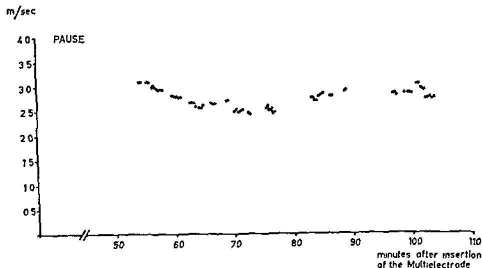


FIG 17 *The effect of pauses on propagation velocity* There is a continuous decrease of the velocity After a pause the velocity is increased in comparison to the value immediately before the pause but there is a more pronounced decrease during the first few seconds of activity The increase in velocity after a pause seems to be correlated to the length of the pause and to the decrease from the initial value The duration of the pauses (indicated with heavy lines) were 28 35 35 75 55 115 and 150 seconds

The most pronounced effects of pauses were seen in cases where the experiments had proceeded for many minutes and the velocity in the fiber had decreased

If the needle was inserted during muscular rest and the activity did not commence until 30–50 minutes afterwards the velocity decrease curve was of the same type as when the activity started at the time of the insertion of the needle The slope of the curves during the first 10 minutes (4 cases) 30–50 minutes after the insertion of the needle was in all cases steeper than the slope during the period between the 30th and 40th minutes of activity in any of the curves obtained in the ordinary way There was no significant difference ($p>0\ 45$) in decrease in velocity during the first 3 minutes of activity in the two types of experiments (Fig 18 Table 1)



Fic 18 *Initial pause* One of the experiments Activity was started 49 minutes after the insertion of the Multielectrode The decrease in velocity seems to be about the same s when the activity is begun at the insertion of the needle (fig 15)

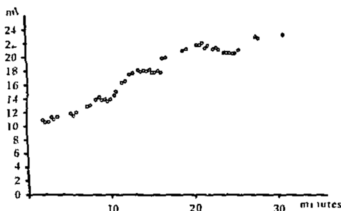


Fig 19 *The action potential amplitude* The first 32 minutes of one of the experiments (totally 38 minutes) presented in Fig 15 (▲)

D *Action potential amplitude*

The amplitude of the action potential usually increased during the ex periment sometimes as much as 120 % (Fig 19) There seemed not to be any relationship between the decrease in propagation velocity and the increase in action potential amplitude The phenomenon is under further study

TABLE 1

The decrease in propagation velocity in different muscles and after an initial paus

Muscle	Number of fibers	Initial propagation velocity	Propagation velocity after 3 minutes	Decrease in velocity during the first		Decrease ii in the inter minutes a start of
				3 minutes	10 minutes	
		m/sec	m/sec	m/sec	m/sec	m/sec 10 mii
Biceps brachii	10	3 37±0 67	2 92±0 63	0 45±0 15	1 09±0 43 range 0 64–1 82	0 22±0 1 range 0 1
Biceps brachii Activity started 30–50 minutes after the insertion of the Multielectrode	7	4 03±0 51	3 61±0 50	0 41±0 17	0 47 0 72 1 13 3 08	
Extensor digitorum communis	14	3 34±0 69	3 09±0 63	0 28±0 15	0 85±0 37	
Frontalis	1	2 20	2 19	0 01	0 05	

DISCUSSION

Only a few reports of measurements of propagation velocity in muscl and nerve fibers during prolonged activity have been found in the literature Hakansson (1956) noticed that there was a small continuous decrease ii conduction velocity in isolated frog muscle fibers and with a stimulatioi frequency of about 10 impuses *per hour* the velocity after two hours was 95–90 % of the value after one hour and 85–80 % after three hours Nc figures were given for the decrease during the first 45 minutes of the experi ment He also reported that "with more frequent stimulation there was a more rapid decrease in conduction velocity" The continuous alteration ii velocity was ascribed to changes in ion balance when the fiber was kep in an artificial medium Gerard & Marshall (1933) showed that the propa gation velocity in frog sciatic nerve and dog phrenic nerve within a quarte of an hour decreased to an equilibrium level less than two thirds of the normal at a stimulation frequency of up to 300 impulses/sec The initial values were reached after about 15 minutes of rest Bullock (1951) reports without further comment that the conduction rate in nerve fibers can d cline tc 50 % of the normal before failure

Many factors are known to influence propagation velocity and these might change during activity, but their relative importance in the present experiments cannot easily be predicted

If the change in mean velocity, found with the Multielectrode, were valid for the whole length of the muscle fiber it could be regarded as ' exhaustion It is known from the literature that myelinated nerve fibers and squid axons can carry action potentials at high frequency for a long time When muscle fibers are stimulated with 50–500 impulses/sec they fail to respond with a conducted action potential after one to several seconds Bergmans (1959) ascribed this to reduction in energy stores but Luttgau (1964) showed that *when the fibers were made non-contractile by continuous stimulation or by* metabolic inhibitors the extracellular action potentials showed only little "fatigue" and the fibers behaved like nerves and were able to conduct action potentials over long periods of time at high frequencies Luttgau assumed action potential "fatigue" to be due to processes connected with the contraction and its activation

It has been shown that the membrane potential and internal resistance in frog sartorious muscle change during prolonged stimulation (Schanne Kern & Schafer 1962) The membrane potential decreased by 12 % and the inter val resistance increased by 17 % It was also shown by Hodgkin & Huxley (1947) that there was a potassium ion leakage from active nerve fibers and that this was associated with changes in membrane properties In isolated nerve Keynes & Lewis (1951) found a gain in sodium ions and leakage of potassium ions already in the resting fibers and still more in stimulated fibers

Even if there is support in the literature for the assumption that part of the decrease in velocity found in the present experiment might be present all along the fiber, the results in Chapter 5 showed that a local velocity decrease in the vicinity of the needle cannot be entirely excluded The following factors could possibly be responsible for this

1) Mechanical damage of the fiber caused by the Multielectrode
2) Disturbance of the electrical field by the physical presence of the needle Multielectrode
3) Changes in the extracellular ion composition

Ad 1 The action potentials recorded from fibers lying close to the needle (as judged from amplitude rise time voltage decrement curves propagation velocity) and from fibers at some distance from the needle were in the main of the same shape If there hade been some mechanical damage to the close lying fiber, there ought to have been an "abnormality" in the potential form Those action potentials described on page 42 as disappearing during the first minute of activity were ascribed to damaged fibers Such recordings were never used for measurements Usually several thousands of

action potentials could be recorded before the fiber was lost for one of the reasons mentioned on page 42 It seems likely that the fibers under study were not damaged

Ad 2 The presence of the needle might alter the external resistance in the vicinity of the fiber due to its metal shaft or to the surface of the insulator surrounding the electrodes As the fiber passed the needle approximately in the middle of the epoxy resin area and the distance from the recording electrode to metal was 500 μ perpendicular to the fiber and 200 μ along the fiber, it seems reasonable to regard the effect of the needle metal on the electrical field of the fiber as negligible Because the electrodes are small (the sum of the electrode surfaces occupies about 6 % of the insulator area) their effects as field deformers might also be disregarded This assumption is also supported by the fact that the "voltage decrement curves" obtained with the two methods in Fig 5 did not differ (Fig 8) indicating that the surfaces of the electrodes did not appreciably disturb the electrical field In the actual experiments the recorded fiber was in the close vicinity of the needle only on one side and over a short distance as the diameter of the needle is 0 6 mm For reasons of symmetry an insulator covering half the circumference of a fiber might double (but not more) the external resistance A flat infinite insulator just touching the circumference cannot possibly increase the external resistance very much

However in this discussion only *changes* in the external resistance are of interest (for discussion of the absolute value see page 50) and a possible effect of the insulator ought to be fairly constant during the course of an experiment

When the relation between the electrical conductance of the medium and propagation velocity has been studied by different authors (for example Pond 1921) this has been done by changing the chemical composition of the fluid bathing the tissue The objection could be raised to these experiments that the velocity might have changed as an effect of the alteration in ionic composition independent of the change in external resistance Hodgkin (1939) studied the effect of the influence of external resistance upon the propagation velocity by changing the geometry of the volume conductor around the fiber thus keeping the ionic composition constant When the crab axon was lifted from sea water into paraffin liquid the impulse velocity decreased by about 20 % (12 5–28 8) When a squid axon was lifted from sea water to air the velocity decreased by 53 % (48–58 %) The velocity of the squid giant axon in the whole nerve in situ was approximately the same as when this axon was in a large volume of sea water It thus seems likely that the disturbance of the electrical field around the muscle fiber due to the presence of the needle has negligible effects on the propagation velocity

Ad 3 When the needle was inserted in the muscle, a number of muscle fibers and lymph and blood-capillaries were damaged and the ion composition in the vicinity of the needle changed from normal As the fiber under study was assumed to be close to the electrode (within 200 μ from the electrode surface, cf Fig 8) the abnormal extracellular fluid surrounded a part of the fiber and could perhaps change the propagation velocity locally

Another possibility is that the normal transport of ions and metabolites was disturbed because of the presence of the Multielectrode This effect ought to be more pronounced at strong activity than at slight

The extracellular fluid could thus theoretically have changed in two different ways

A The ionic composition of the extracellular fluid became abnormal at the insertion of the needle and was gradually normalized during the experiment

B The ionic composition of the extracellular fluid was initially normal but altered during the experiment

A *Abnormal extracellular fluid at the beginning of the experiment*

If the extracellular potassium ions from injured fibers poured out immediately at the insertion there could theoretically have been an initial depolarisation generating a conducted action potential if the depolarisation was fast or if the depolarisation was slow making the fiber inexcitable (Jenerick & Gerard 1953) The difficulties in obtaining any recording during the first 10–30 seconds after the insertion of the Multielectrode (page 20) might have been caused by such a depolarisation

If there was an initially changed composition of the extracellular fluid due to the insertion of the needle this could be assumed to be continuously normalized The absorption of ions should proceed even during muscular rest although perhaps somewhat more slowly than during the slight activity normally performed during the experiments

The experiments where activity was not begun until 40–50 minutes after the insertion of the needle indicated that the decrease of propagation velocity did not start until the fiber was activated This makes it unlikely that the possibly change in extracellular ion composition caused initially too high a velocity which continuously decreased toward normal values

B *Normal extracellular ion composition at the beginning of the experiment*

If the extracellular fluid were normal at the beginning the propagation velocity decrease ought to be caused by change in the fiber properties or in the composition of the environment around the fiber or to both of these factors Owing to the presence of the needle the normal transport of ions

substrate and metabolites around the fiber could be altered because of interference with blood-capillaries and lymph-capillaries and the possible general effect upon the propagation velocity during activity might be enhanced in the vicinity of the needle Such an effect could be cumulative during prolonged activity At high frequency the effect ought to be more pronounced than during low frequency This assumption is supported by the findings in the experiments where the effect of different frequencies was studied and the experiments where the activity was stopped for some seconds after many minutes of activity If this hypothesis of a change in the extracellular ion composition during activity is true the propagation velocity value measured in the beginning of the activity most closely reflects the velocity as it would have been if no Multielectrode were present

PROPAGATION VELOCITY OVER LONG DISTANCE

SUMMARY

1 The brachial biceps and extensor digitorum communis muscles were used

2 Attempts to record activity from one and the same fiber with two needle electrodes were not successful neither at electrical stimulation nor at volutary contraction

3 A nerve fiber was stimulated and the latency between the stimulation pulse and the muscle fiber action potentials was measured

4 The latency decreased during continuous activity Under the assumption that the nerve propagation time and the neuro muscular transmission time were constant during activity it could be calculated that there was a decrease in propagation velocity not only in the vicinity of the Multielectrode

INTRODUCTION

In Chapter 4 it was found that the propagation velocity measured with the Multielectrode technique decreased during the course of the experiment in all the fibers and that this decrease seemed to be positively correlated to the degree of activity At rest the velocity recovered The decrease in velocity proceeded most rapidly during the first 15 minutes of continuous activity after which time the velocity reached more constant values an equilibrium was reached between the decrease and the recovery

A decrease in velocity of the order of 30–50 % observed in some of the experiments (Fig 15) has not been reported in the literature There are theoretical reasons for a certain decrease in velocity during continuous activity (page 47) but a local change in the vicinity of the Multielectrode due to its presence cannot *a priori* be excluded

The insertion of the needle might cause alteration of the chemical milieu of the fiber under study so that the values obtained with the Multielectrode would not represent the propagation velocity of the whole muscle fiber

The aim of the present chapter is to investigate whether the observed decrease in velocity is generalized or local

METHODS

Electrical activation

One recording electrode The *1+13* Multielectrode was used for recording and a bipolar needle electrode (DISA 13K14) for stimulation The stimulating electrode

was inserted in the distal third of the muscle and the Multielectrode some centimeters proximal to that The pulse width was 60 μsec the stimulation frequency 2–10 impulses/second By frequent adjustment the stimulation strength was kept at the minimum level at which all stimuli would still be effective (in the following called "threshold stimulation")

Measurements were made of the propagation time of the action potential over the Multielectrode and of the latency between the onset of the stimulus pulse and the base line intersection of the action potential picked up by one of the electrodes in the Multielectrode

The distance between the stimulating and recording sites was estimated from the distance between the needles along the skin the depth of the needles in the muscle and the angle between them The accuracy was not better than ± 2 mm

The experiments were made in the brachial biceps muscle (50 fibers in 5 different subjects) and in the extensor digitorum communis muscle (25 fibers in 4 subjects) The subjects were healthy men 25–35 years of age

Two recording electrodes A technique similar to that described by Buchthal Guld & Rosenfalck (1955*a*) was used in about 10 experiments in the brachial biceps muscle One stimulation electrode (DISA 13K14) was inserted in the distal third of the muscle One recording electrode (DISA 13K51) was inserted some centimeters proximal to that and the Multielectrode some centimeters still more proximally A position close to one and the same activated fiber was sought for the two recording needle electrodes

Voluntary activation

Recordings from fibers belonging to the same motor unit with two needle electrodes The *1+13* Multielectrode was inserted in the muscle and positioned so that the propagation velocity could be measured from one fiber This action potential triggered the sweep of the oscilloscope Another recording electrode (usually a concentric needle electrode DISA 13K14 sometimes another Multielectrode) was inserted some centimeters more distally in the muscle and was positioned so that an action potential was recorded which was "tied to the first potential

Propagation time was measured both between the potentials recorded from the two needle electrodes and with the Multielectrode

RESULTS

Electrical activation

One recording electrode When using one stimulating and one recording electrode the mean value of the time between the stimulus and the muscle response (latency) could vary because of technical factors

1 By moving the needles in the muscle i e changing the distance between the recording and stimulating electrodes, the latency could be changed many milliseconds

2 By increasing the stimulation strength from threshold value it was possible to shorten the latency up to 1 millisecond

When however, the needles were kept in a fixed position in the muscle and threshold stimulation was used a change in the latency was still seen during continuous activity

Often many potentials were recorded (belonging to the same motor unit or to different motor units with approximate the same stimulation threshold of the nerve) For technical reasons only the first of the single fiber action potentials in such a complex could be used

In 6 out of 15 cases the latency increased by 1000–3000 μsec (11–23 % of the initial value) during 7–29 minutes In 6 cases the increase was less than 1000 μsec (0–10 %) during 5–12 minutes In one case no change was seen during 6 minutes and in one case the latency decreased 200 μsec (2 %) during 60 minutes of activity

In 2 cases the relative increase in propagation time measured over the Multielectrode was approximately the same as the relative increase in latency but in most experiments far more (Table 2)

There was a variability in the latency between consecutive discharges of the order of 10–30 μsec (SD) The variability of the propagation time over the Multielectrode was of the order of 0 05–0 1 μsec (SD) which is nearly the same as the inaccuracy of the time measurements (page 18)

Two recording electrodes In the experiments performed with the technique similar to that described by Buchthal Guld & Rosenfalck (1955*a*) with electrical stimulation it was possible to record single fiber action potentials from the two recording electrodes Some recordings were obtained which fulfilled the criteria given by Buchthal Guld & Rosenfalck for measuring propagation velocity namely a) the action potentials disappeared at the same stimulating voltage when it was decreased and b) there was a rectilinear relationship between the arrival time and the distance from the stimulation electrod However during 50 hours of experiments it was never possible to get a constant interval between the action potentials recorded from the two electrodes

In the experiments with one stimulating and one recording electrode there was a variability in latency of the order of 5–15 μsec at regular stimulation frequency independent of the distance between stimulating and recording electrodes The variability was assumed mainly to be caused by a variability in the neuro muscular transmission time If two electrodes recorded ctivity from one and the same fiber the variability in latency should be even smaller When the electrode however recorded from different stimulated fibers two motor end plates were involved giving a larger variability In the actual recordings the variability was of the order of 10–30 μsec indicating that the electrodes recorded activity from different fibers

Voluntary activation

In 19 experiments with voluntary activation the recordings were made with two electrodes separated by about 20 millimeters In all cases (at regular frequency about 30 μsec SD, at irregular frequency even more) there was a *small variability* in the time interval between the potentials recorded from the two needles This variability seemed usually to be random but in some cases it was influenced by the intervals between the consecutive discharges In no case during more than 100 hours experiment could it be ascertained (for criteria see page 58) that the two electrodes recorded action potentials from the same fiber, and therefore each experiment was not extended for a long period of time

DISCUSSION

Direct muscle stimulation

One way of testing whether the propagation velocity measured over the Multielectrode was valid for a longer distance along the muscle fiber would have been to stimulate the muscle fiber some centimeters from the recording electrode and to measure the time lag between the stimulus and the record d action potential Direct stimulation of single muscle fibers proved however not to be feasible (Chapter 3)

Electrical stimulation of intramuscular nerve twigs

Recording with one electrode The latency between stimulus and action potentials includes the delay from onset of the stimulus to the initiation of an action potential the propagation time along the nerve the neuro muscular transmission time and the propagation time along the muscle fiber As the distance from the motor end plate to the recording electrod was unknown as was also the time taken for the impulse to propagate along this part of the muscl fiber the velocity of the impulse could not be calculated from these experiments When an "apparent velocity" was calculated this was always higher than the velocity over the Multielectrode This was partly due to the fact that those action potentials had to be chosen for recording which had the shortest latency Some other potentials in the response had more than twice the latency of the shortest one

To get an id a of which apparent lengt (L_{app}) that might correspond to the increase in latency if the propagation velocity was assumed to decrease as much all along the fiber as over the Multielectrode the following analysis was made It was assumed that all factors contributing to the total latency were constant exc pt for the propagation time along the fiber "L_{app} " then was obtained from the following equation

TABLE 2

Measurements of latency between stimulus and an action potential recorded with the Multielectrode and the propagation time over the Multielectrode

Duration of activity	Stimulation frequency	Measurements between two needles			Measurements over the Multielectrode			Distance between the needles	
		Initial latency	Change in latency		Initial prop time	Change in prop time		Actual	L_{app}
Minutes	impulses/sec	µsec	µsec	%	µsec	µsec	%	mm	mm
8	5	24 500	3000	12	118	45	38	86 7	15 4
21	5	10 770	1587	15	106	18	17		48
33	2 5 10	10 580	1370	13	91	69	76	40	3 0
7	5	4784	1000	23	57	13	23	20	16
29	5	7900	1000	12				70	
17	1–10	8800	1000	11				38	
21	2 5 10	15 520	850	5 5				60	
5	5	6200	500	8				32	
9	5	6023	440	7	72	71	15		8 8
5	5	6601	18	0 0	111	–24	–21		
6	5	29 350	0	0	150	71	48	104 7	
60	5	10 059	–200	–2					

$$\frac{L_{app}}{L_{ME}} = \frac{\Delta \text{ latency}}{\Delta \text{ prop time}_{ME}}$$

where L_{ME} = interelectrode distance in the Multielectrode
Δ latency = change in latency and
Δ prop time$_{ME}$ = change in propagation time over the Multielectrode

L_{app} was found to be 3 to 48 mm (Table 2) In only one case of 3 where a comparison was possible there was a reasonably good agreement between these values and the actual distance between the needles In the two other, L_{app} was much too small If the propagation time in the nerve and the transmission time in the neuro muscular junction did *not change during* the experiment these figures indicate either that the impulse had travelled along the muscle fiber for only a short distance and that the decrease in velocity was generalized for this distance or if the length along the muscle fiber was longer than calculated that the decrease in velocity over the long distance was l ss than that over the Multielectrode In Fig 20 one method for calculation of the L_{app} is presented

The extreme difficulty of measuring the propagation velocity over the Multielectrode in these experiments must however be stressed The experimentor had to keep two electrodes the Multielecetrode and the stimulating

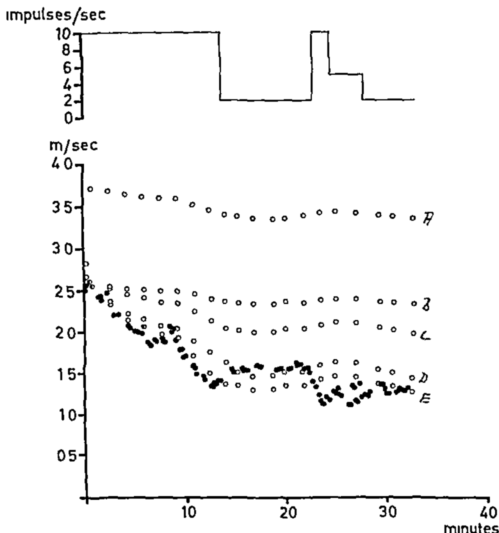


Fig 20 *The propagation velocity decrease over the Multielectrode (▼) and the decrease of the apparent velocity over a long distance (A–B)* Note that the decrease has a different slope at different stages of the experiment because of different stimulation frequencies *A* is the values of apparent velocity that is obtained when the distance between the stimulating and recording needle (40 mm) is used for calculation In *B* the propagation distance has been calculated to such a value (27 7 mm) that the initial velocity is equal to that measured over the Multielectrode In *C D* and *E* a fixed time (6 9 9 2 and 9 6 msec respectively) has been subtracted from the latency and thereafter the propagation length calculated to a value that should give the same initial velocity as over the Multielectrode (10 4 and 3 mm respectively) The time subtracted in the three cases should correspond to the propagation time in nerve and the neuro muscular transmission time

electrode simultaneously in the correct position to within a few micron Another reason for uncertain results was that the recorded action potential was influenced from other action potentials This disturbance might change during the experiment when there was a continuous change in the stimulation threshold

The change in latency was also analysed in the following way The change in the apparent velocity (total latency divided by that calculated distance which initially gave a velocity of 3 5 m/sec) was compared to the decrease in velocity obtained over the Multielectrode in voluntarily activated fibers given in Table 1 The slope of the decrease curve of the apparent velocity was seen to be approximately the same throughout the experiment On an average the decrease in apparent velocity expressed as the total decrease during 10 minutes of activity was 0 25 ± 0 28 m/sec which is significantly less than the normal decrease 1 09 ± 0 43 (p < 0 01 Table 1) obtained over the Multielectrode When also a constant time value (corresponding to propagation time in nerve and transmission time in the neuro muscular junction) was subtracted from each latency value the relative decrease in apparent velocity was higher than before However such a calculation cannot be performed with any degree of accuracy because not even a rough estimate of the propagation time in the nerve fiber can be made

Further factors which could be an influence are in addition A systematic change in the starting point of the action potentials and continuous change of the propagation time along the nerve fiber and changing neuro muscular transmission time

The decrease in latency noticed at increasing stimulation strength is a phenomenon that has been reported before and is supposed to be due to a change in the starting point of the action potential (Gerard & Marshall 1933) In the present experiments the stimulation strength was continuously adjusted to threshold and therefore it was assumed that the impulse had started from approximately the same site on the nerve fiber

A change in neuro muscular delay in skeletal muscle under normal conditions during continuous activity has not been reported in the literature A decrease in transmission time should partly mask an increased propagation time along the muscle fiber An increase in transmission time should make the decrease in velocity along the muscle fiber less than calculated In the present experiments neuro muscular transmission block was never seen (as a sign of the ultimate increase in transmission time)

Two recording electrodes It was never possible to record activity from one and the same fiber with two different electrodes Therefore the stimulation was not continued for a long period No further information concerning the

problem of the change in propagation velocity in a single muscle fiber would have been obtained when different action potentials from fibers were recorded with the two electrodes

Voluntary activation

Two recording electrodes In these experiments the problem of uncertainty in the starting point on the nerve did not arise For the calculation of propagation velocity it was however, necessary to ascertain that the electrodes recorded action potentials from the same fiber This was tested by comparing the short time variation in propagation velocity over the Multielectrode with the variation in "apparent velocity The propagation velocity for each action potential in a muscle fiber is influenced by the time interval to the preceding impulse (Chapter 6) If the electrodes in the present experiments were recording from one and the same fiber the interval dependence of the propagation velocity should be the same if it was calculated over the Multielectrode or between the two needles If however the electrodes recorded from two different fibers the interval-dependence in the calculated velocity would not necessarily be the same for the short and long distances In the two fiber case the propagation time measured between the electrodes is equal to the difference in the conduction time from the common branching point in the nerve to the respective electrode Theoretically there are several reasons for short time variations in this conduction time

1 Uncertainty in propagation across the branching points of the terminal nerve (cf Krnjevic & Miledi 1958)
2 Variability in the propagation velocity in the nerve fiber
3 Variability in the neuro muscular transmission time (Ekstedt 1964)
4 Variability in the starting point in the motor end plate (del Castillo & Katz 1956)
5 Variability in the propagation velocity along the mucle fiber (Chapter 6)

Some of these factors are dependent on the innervation rhythm but the contribution of each factor to the total variability in the latency cannot be predicted Therefore when the two electrodes were recording from two irregularly innervated fibers belonging to the same motor unit the velocity interval dependence should not regularly be the same when it was measured in one fiber over the Multielectrode as when it was measured between the needles However even when recording from two fibers this interval dependence might be present provided that that there is a considerable difference in propagation length for the two fibers In this case however the factors 1 to 3 above must be superimposed (page 77) but still the variation in propaga

tion velocity between consecutive impulses might be similar over the long and the short distance Thus the presence of the same velocity interval dependence over the long and short distance speaks in favour of a one fiber recording and the absence indicates recording from two fibers In no case was the same VRF found over the long and short distances

In this investigation it has been shown that it is extremely difficult to measure the propagation velocity in a single fiber over a long distance *in situ* As a matter of fact it was not possible in any case to ascertain that the used electrodes recorded action potentials from one and the same fiber in any case Therefore it has also been difficult to study the change in propagation velocity during prolonged activity The results indicate that there is a decrease in velocity in the whole fiber during activity, but that the decrease over the Multielectrode is more pronounced

VARIABILITY IN PROPAGATION VELOCITY FOR CONSECUTIVE DISCHARGES IN A FIBER

"THE VELOCITY RECOVERY FUNCTION"

SUMMARY

1 The interspike interval dependent variability in the propagation velocity was studied

2 The experiments were performed with voluntary activation or with electrical stimulation of intramuscular nerve twigs

3 The general appearance of the interval velocity curve was obtained with double pulse stimulation For the time interval of about 3–10 msec after a preceding impulse the velocity was *subnormal* For a longer time interval the velocity was *supernormal* with maximum (112 5–124 %) at the interval 8 50 msec After the maximum there was an asymptotic decay After 500 msec some supernormality still remained This curve is called the Velocity Recovery Function (VRF)

4 When stimulating with a train of pulses the effect of each impulse was added to the remaining effect of the preceding impulses

5 At voluntary contraction where the innervation frequency was irregular this effect causes an interval dependent variability of the propagation velocity

6 A method has been developed for calculating the descending part of the VRF from the interval dependent velocity variations

INTRODUCTION

In experiments with voluntarily activated muscles it was seen that the variability in propagation velocity was greater than could be ascribed to inaccuracy in measurements This variability was studied with the ultimate aim of finding out whether this would give some information about the membrane processes of the muscle fiber under study

METHODS

The experiments were performed in the brachial biceps and the extensor digitorum communis muscles in 20 healthy young men Four different methods were used

1 Double pulse stimulation

When stimulating electrically the bipolar stimulating needle (DISA 13K14) was placed distally in the muscle and the Multielectrode (1+13) some centimeters more proximally Square pulses of a duration of 60 μsec were used The strength

of both the conditioning pulse and the test pulse was just above threshold for the nerve fiber (the muscle fiber was usually activated via its nerve Chapter 3) so that spontaneous fluctuations in excitability did not make stimuli ineffective The interval between the pulses (conditioning to-test interval) was varied and the interval between the different pairs of pulses was always more than 2 seconds

During the 5–100 msec after the conditioning pulse the nerve membrane was hyperexcitable and therefore the test pulse must be of lower voltage than the conditioning pulse When using the same stimulation strength for the two pulses usually more nerve fibers were activated by the test stimulus This was most pronounced when the pulse interval was 5–50 msec When the interval was less the testing pulse had to be stronger than the conditioning one

In most cases it was possible to determine from the form of the action potentials whether they were generated from the same fiber or not

Propagation velocity was measured from the action potential initiated by the test pulses every second of which was preceded by a conditioning pulse

The time intervals between the conditioning and the test action potentials was measured b tween the base line intersections of the rising phases This interval differs somewhat from the interval between the stimuli because of varia tions in neuro muscular delay latency in the starting point and different propaga tion velocities for the two pulses (Farmer Buchthal & Rosenfalck 1959)

2 *Stimulation with trains of pulses*

The same general techniques as above were used The fibers were stimulated with different frequencies in trains with a duration of 10–12 seconds and during the following 30 seconds the fiber was stimulated with a single shock every two seconds The frequency within the train was 5 10 15 or 20 impulses/sec

3 *Stimulation with irregular stimulation rhythm*

A comparison of the variability of propagation velocity in different fibers was made by electrical stimulation with an irregular and exactly defined rhythm (programmed stimulation) The experiments were performed in the following way The discharge rhythm of voluntarily activated action potentials was recorded on a tape recorder (page 19) The tape was played back and the pulses triggered the stimulator The propagation velocity over the Multielectrode was measured in the ordinary way

4 *Voluntary activation*

Propagation velocity was measured with the Multielectrode technique The subject was asked to make the innervation rhythm irregular Aided by the sound of the action potentials in the loadspeaker some of the subjects could at will produce certain types of irregular rhythms e g a required number of discharges in trains (cf Basmajian 1963) The mean frequency was varied from a few up to about 10 per second At this frequency it was sometimes still possible to record single action potentials without too much disturbance from other motor units The propagation velocity of each impulse and the interspike interval were determined

For the analysis of the VRF when the propagation velocity was measured over a long distance ten experiments have been performed according to the

technique on page 51 i e the *1+13* Multielectrode recorded activity from one muscle fiber and a concentric needle electrode inserted some centimeters proximally recorded from another fiber belonging to the same motor unit The recordings were analysed according to the principles on page 68

The points in the figures indicate the mean propagation velocity over a 10 second period when not otherwise stated

RESULTS

Double pulse stimulation

It was found that the difference between the propagation velocity of the action potential elicited by the test pulse and the conditioning pulse was correlated to the interspike interval in a manner shown in Fig 21 This curve is referred to as the *Velocity Recovery Function* (VRF)

The VRF was different for different fibers and changed during certain experimental conditions (see Chapters 7 and 8) Only five of the VRF curves obtained with the double pulse technique were of reasonably good quality but even these are uncertain because of the experimental difficulties

A supernormal phase of the VRF was seen in four of the five curves In

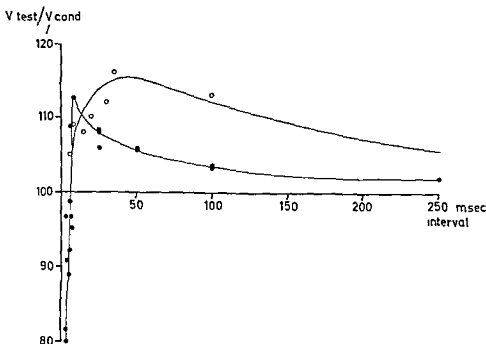


FIG 21 *Double pulse stimulation* Two different curves are shown Abscissa The time interval between the conditioning and testing action potentials Ordinate The velocity of the testing action potential (V_{test}) 100 % =the velocity of the conditioning action potential (V_{cond}) The curves are fitted by eye The upper curve is drawn towards a measured value at 500 msec not shown in the figure

the fifth case no supernormal phase was present. At the end of the refractory period (about 2–4 msec) the propagation velocity of the second action potential was about 20 % lower than that of the first. At 6–10 msec interval the two action potentials had the same propagation velocity. In the four experiments where there was a supernormal phase the curve had a maximum at an interval of 8–50 msec where the second pulse propagated 12.5–24 % faster than the first pulse. The supernormality then gradually decreased. At 50 msec the supernormality was 5, 5, 5.5, 14, 17 and 24 % in six cases and had decreased to 50 % of maximal value when the interval was 100–300 msec. After 500 msec the supernormality was less than 2 %.

Trains of pulses

Thirty fibers in 5 subjects were investigated. When stimulating with trains

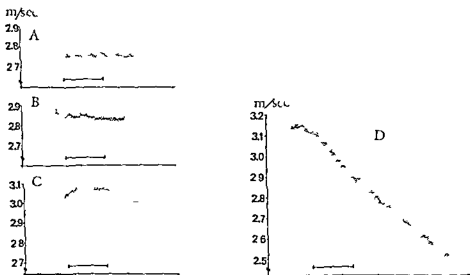


FIG. 22. *The effect of stimulation with trains of pulses on the propagation velocity over the 1+13 Multielectrode.* The basal stimulation frequency is about 1 per 2 seconds in each case. *A*. 5/sec. Velocity increases 0.05 m/sec. The plateau value is fairly constant. *B*. 10/sec. Velocity increases 0.13 m/sec. The plateau shows a slight tendency to decrease during the train. There is a slight undershoot of the velocity after the train. *C*. 15/sec. The velocity increases 0.33 m/sec (extrapolation because the needle position is not correct during the first 3 seconds). The decrease of the plateau during the trains is pronounced as is the undershoot after the train. For the first second of the pulse train all values are plotted, then only every third. *D*. 20/sec. The velocity increases 0.36 m/sec. After 40 seconds of stimulation the velocity has decreased to 2.52 m/sec which is 0.63 m/sec less than the highest value during the train and 0.27 m/sec below the resting value before the train. After the stimulation the velocity tends directly towards the initial value without any further decrease. For the first 2 seconds of the pulse train all values are plotted, then only every second.

Calibration in each case 10 seconds.

of pulses there was an increase in propagation velocity during the first few seconds from a basal level to higher values which decreased somewhat during the train (Fig 22), The decrease was more pronounced for higher stimulation frequencies than for lower and for each frequency the decrease was more pronounced later in the experiment when the fiber had been stimulated for many minutes With the stimulation frequencies 5 10, 15 and 20 impulses /sec the velocity was increased 0 03, 0 15, 0 30 and 0 40–0 60 m/sec respectively The maximum value was reached after 400–600 msec of stimulation for all frequencies (Fig 22) At the end of the pulse train the velocity decreased as much as it had increased in the beginning of the train i e propagation velocity fell below the basal level After 5–10 seconds the propagation velocity had reached the basal level again

Stimulation with irregular rhythm

About 20 fibers in 5 subjects were investigated

Similar results as in the experiments with double pulses were obtained with the irregular innervation frequency The VRF could, however, not be directly determined as with the double pulse technique It was not only the last preceding impulse that influenced the propagation velocity but earlier pulses as well When the same stimulation pattern was used for different fibers the propagation velocity varied in the same manner for the different fibers

Voluntary activation

The velocity interval dependence could be seen during the experiments by direct observation of the Time Interval Counter where a short interspike interval as a rule was accompanied by a short propagation time The interval depend nce of the propagation velocity has in this way been observed in about two thirds of the voluntarily activated fibers ever since the phenomenon was first noticed that is to say in many thousands of fibers in the 170 experiments which have been performed

Closer analysis was made in one of the following two ways

a) The propagation velocity for each impulse was plotted against continuous time From these diagrams it was possible to determine whether the VRF had a supernormal part (after a short interspike interval the velocity was higher, after a long interval it was lower Fig 23) a subnormal part (the velocity was lower after a short interval higher after a long Fig 34 C) or whether the functions was near the basal level for the interspike intervals used (no variability in propagation velocity in spite of irregular frequency Fig 34 B)

b) In other cases where at least 200 consecutive discharges and the corre

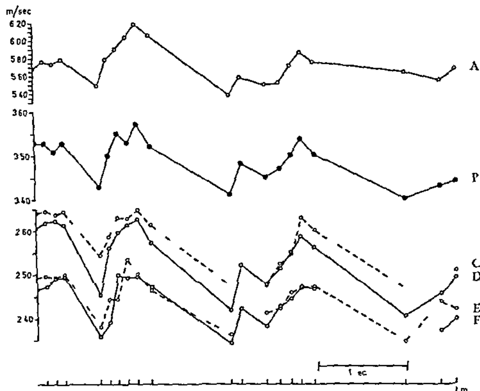


FIG 23 *The velocity interval-dependence at irregular innervation frequency* B is a recording during voluntary activation The innervation frequency was recorded on a tape recorder which could be played back into the stimulator *D* and *F* shows the variability thus obtained with another fiber in the same subject and with the same stimulation pattern as in B Both *D* and *F* begin approximately with the 350th action potential measured in the respective recording Action potentials number 1–200 were used for calculation of the Velocity Recovery Function and the propagation velocity values were computed from the time intervals and shown in *C* and *E* Curve *A* is another fiber

sponding interspike intervals had been recorded and no change in the mean velocity was detected the analysis was made according to the description on page 68

With voluntary activation time intervals from 50 to 500 msec were normally obtained Sometimes however even shorter interspike intervals were recorded after a resting period of 3–10 seconds following prolonged activity there were sometimes double discharges with an interspike interval of about 6–10 msec A similar phenomenon was described by Standaert (1963)

With the analysis described the velocity interval dependence was usually

found in rested fibers but in some cases there seemed only to be a random variability in velocity No systematic differences could be noticed between the recordings where the VRF did have a supernormal phase and where it did not neither with regard to the slope of the velocity decrease curve during activity, action potential amplitude, innervation frequency, the type of muscle nor to position of the needle in the muscle With a sweep of 20 μsec/cm and amplification of 1 mV/cm on the oscilloscope screen the action potential form did not indicate any disturbance from adjacent fibers but nevertheless action potentials from other fibers of the motor unit might have distorted the propagation time interval (Fig 24)

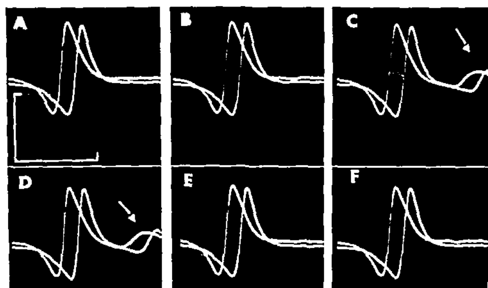


FIG 24 *Interference from another action potential on the propagation velocity measurement* Six consecutive discharges from a long series of recordings In A B E nd F the propagation time is 84 4 84 8 84 8 and 84 5 μsec (propagation velocities 2 855 2 842 2 842 and 2 852 m/sec) In C and D an action potential from another motor unit appears (the arrows) in the late parts of the actual action potentials apparently without influencing the potential form but giving propagation times 86 0 and 85 7 μsec (2 802 and 2 812 m/sec) Calibration 2 mV and 500 μsec

During continuous activity for a long period the VRF sometimes changed In some fibers the supernormal phase decreased or even disappeared after 20 minutes of activity In 2 of 30 fibers the supernormal phase was changed towards a subnormal phase for the normal interspike intervals (50–500 msec) In none of 50 cases could any changes be detected during the first 10 minutes of activity

In the experiments with two recording needle electrodes the latency between the action potentials recorded from the two needles varied from ±25 μsec to ±39 μsec (SD) in 7 cases when the interelectrode distance was about 10 mm and from ±50 μ to ±70 μsec (SD) in 3 cases when the interelectrode distance was about 20 mm The innervation frequency was irregular The variability in propagation time over the Multielectrode varied between ±1 μsec and ±2 μsec (SD) An "apparent" velocity between the two needles was calculated The actual distance between the needles was not used but such a distance as gave approximately the same velocity as that measured over the Multielectrode The variability in propagation velocity measured both over the Multielectrode and as the "apparent" velocity between the needles was analysed with the method described on page 68 In all of the ten recorded fiber pairs a velocity interval dependence was found both over the Multielectrode and between the two separate needles Since two fibers were involved in the recording and the real propagation length was not known no quantitative comparison between the variability obtained over the Multielectrode and between the two needles has been made In Fig 25 an example

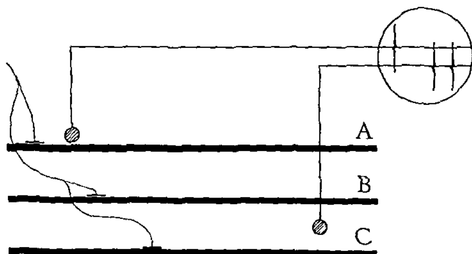


FIG 25 *A recording from three muscle fibers from the same motor unit* Velocity interval dependence was found between action potentials from both fibers A-B and A-C

of experimental condition is given where one of the electrodes recorded activity from two fibers belonging to the same motor unit

The amplitude of the action potentials from 50 fibers was studied on photographs but no supernormal phase was found The amplitude was about 5 % lower at an interval of 10 msec and about normal for the testing pulse

after 300–500 msec With a train of pulses with 10 impulses/sec the amplitude had decreased to a constant value about 95 % of the maximal after about one second

ANALYSIS OF THE VELOCITY RECOVERY FUNCTION (VRF)

As shown in this chapter, the propagation velocity is changed during the recovery phase after an action potential The general appearance of the velocity interval dependence curve was obtained in the experiments with double pulse stimulation However, these experiments were technically very difficult to perform and the results uncertain When the interval between conditioning and testing impulse was changed the threshold of the nerve membrane for the test pulse was also changed This sometimes caused more than one muscle fiber in the vicinity of the Multielectrode to be activated and the value of the propagation velocity often became erroneous

Therefore a method for analysis of the VRF from propagation velocity values obtained at voluntary contraction has been worked out

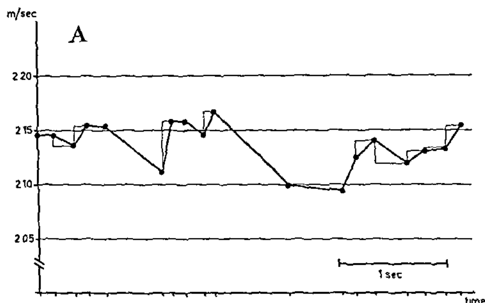
A period of the experiment was selected where the mean propagation velocity value did not seem to change From this period (about 10–60 seconds) 200–400 consecutive propagation velocity values with a variation of normally about ±0 1 m/sec (range) were taken for analysis

The whole course of the velocity recovery curve cannot be analysed when using voluntary activation The interspike intervals (50–500 msec) are only distributed in the descending part of the curve

An attempt to find a function that fitted the data directly (e g an exponential function) was unsuccessful Therefore the following method was applied

Since the steepness of the recovery curve seemed to be dependent on the level above a basal value the propagation velocity values were divided into classes with a width of 0 05 m/sec, e g 3 00–3 05 3 05–3 10 3 10–3 15 m/sec All action potentials within such a class were regarded as "conditioning pulses" The increase or decrease of the velocity of the action potential arriving after the conditioning action potential was plotted against the time interval between the two Usually the intervals ranged from 50 to 500 msec In this way one velocity recovery curve was obtained for each 0 05 m/sec level and all potentials were used once as conditioning once as test action potentials (Figs 26 and 27)

The mathematical expression for the biological processes underlying the VRF are not known To obtain a way of presenting the results and to facilitate further analysis (e g when comparing the VRF from different fibers or when the curves in one and the same fiber were studied during prolonged work during ischemia or at different temperatures) a function that suited the data reasonably well was sought from the many possible



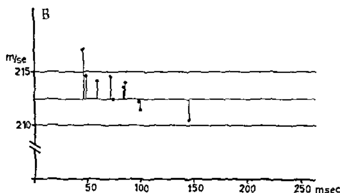


FIG 26 *Principles for the analysis of the Velocity Recovery Function at voluntary innervation* The propagation velocity values are divided into classes In *A* the values between propagation velocity 2 10 and 2 15 m/sec are thus analysed All action potentials with propagation velocity within a certain class are considered as conditioning impulses The change in propagation velocity to the next action potential the test impulse is calculated as well as the time interval to this For each class the change for the test pulse in propagation velocity (abscissa) is plotted against the time interval from the conditioning impulse (ordinate) as made in *B* The time scale is different in *A* and *B*

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The mathematical expression for the biological processes underlying the VRF are not known To obtain a way of presenting the results and to facilitate further analysis (e g when comparing the VRF from different fibers or when the curves in one and the same fiber were studied during prolonged work during ischemia or at different temperatures) a function that suited the data reasonably well was sought from the many possible

The values in each class seemed to be asymptotically approaching a basal level that was roughly the same for the different classes Evidently they should reach the basal value of propagation velocity that the fiber would have had with a very low innervation frequency It could thus be anticipated that the function would have the general form

$$V_t = V_{bas\ 1} + \frac{B}{F(t)} \tag{1}$$

where V_t = the actual value of the propagation velocity at time = t
$V_{bas\ 1}$ = the basal velocity of the fiber, i e the asymptotic value
B = a proportionality factor
F(t) = a function that increases monotonically with time

Different F(t) were investigated and compared in the following way In each class a linear regression analysis was made of ΔV (difference in propagation velocity between conditioning and testing action potential) on 1/F(t) where F(t) was t t, $\sqrt{t}$ and ln*t* The linear regression line was calculated with the method of least squares F(t) = t and F(t) = t did not fit very well However F(t) = $\sqrt[3]{t}$ and F(t) = ln*t* both fitted the data well For comparing the four equations the root mean square difference between the observed values and those calculated was used In nearly all of 30 examined VRF curves this value was least for F(t) = ln*t* (order of magnitude 1 10 -–2 5 10 m/sec) but the function F(t) = $\sqrt{t}$ was almost as accurate The two other functions however, gave values that were 5–10 times higher

Hence the following equation was used

$$V_t = V_{b\ \ 1} + \frac{B}{\ln t} \tag{2}$$

However as already said this function does not represent the whole VRF but only the descending part of it and must not be used for values less than about 50 msec (an interval exceeding that where the VRF has its maximum page 63)

In Fig 28 an example is given from an experiment with voluntary activation of the muscle showing how it was possible to predict propagation velocity values when the spike intervals are known The VRF-curves for the different classes were determined from 200 consecutive readings For the following 200 data the propagation velocity of each impulse was read from

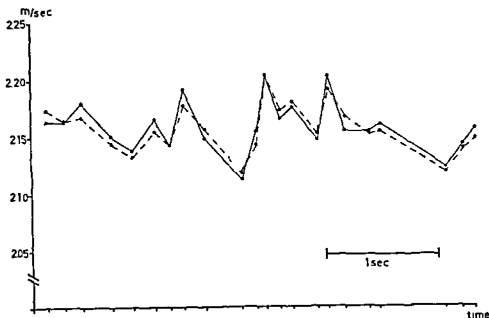


Fig 28 *Prediction of the propagation velocity values by means of the VRF* The points joined by lines represent the propagation velocity values of consecutive action potentials (ordinate) plotted against continous time (abscissa) The VRF was computed for 200 action potentials preceeding the shown ones By means of the function and the interspike intervals the propagation velocities have been computed and shown as the points that are joined by the dashes

the interspike intervals and the corresponding curves There was a good agreement between the calculated and actual values

With the analysis presented two characteristics of the fiber can be obtained the basal velocity (V_{basal}) and a measure of the supernormality

Ideally the VRF of a specific fiber would have been that obtained with the double pulse technique There a conditioning pulse would have a velocity equal to V_{basal} With voluntary activation it was however usually impossible for the subjects to activate the muscle with such a low frequency that V_{basal} was approached Therefore the following procedure was used

The basal velocity was calculated from each class Usually these asymptotic values were approximately the same for the different classes in an experiment Sometimes the V_{basal} calculated for the highest class differed from the others by as much as 0 1 m/sec probably because here the whole curve was based on actual data from very short time intervals Nonetheless the average of the V_{basal} obtained from the different classes was used

TABLE 3

Parameters of the Velocity Recovery Function calculated according to the method on page 68. For some further normal values see Table 4 (the pre-ischemic periods)

Muscle	Experiment and notation	Calculated V_{basal} m/sec	Calculated increase in propagation velocity at 50 msec for the basal class (per cent)
Extensor digitorum communis	796 Voluntary activity	2.8	3.5
	831 Voluntary activity	2.6	1.6
	Same fiber one minute later	2.6	4.5
	809 After 13 minutes of voluntary activity	2.6	8.5
	Same fiber after 57 minutes of activity	2.6	5.4
	1042 After 1 minute of electrical stimulation	2.3	12.3
	After 12 minutes of stimulation	2.2	8.4
	After 21 minutes of stimulation	2.2	10.7
Biceps brachii	810 After 8 minutes of voluntary activity	2.3	7.1
	After 18 minutes of activity	2.0	-1.8
	823 Voluntary activity	3.9	5.0

A measure of the supernormality was obtained in the following manner. The propagation velocity at an interval of 50 msec for each class was read from the fitted curves and was plotted against the mean value of the classes. A linear relationship was found. As the V_{basal} had been obtained as described above, a velocity value at $t = 50$ msec could also be calculated for a basal velocity class. The procedures are presented in Fig. 29. Some of the results are presented in Table 3.

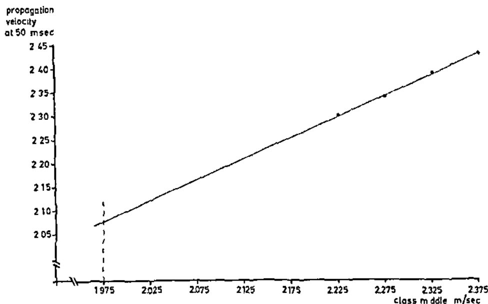


FIG 29 *An attempt to find the parameters for the Velocity Recovery Function for the basal class of the fiber analysed in fig 27* The propagation activity value for line firm interval 50 msec (see equation 2 page 71) (ordinate) was plotted against the class middle of each Velocity Recovery Curve in fig 27 For the class middle = V_{basal} (equation 2 page 71) (i e in this case 1 975 m/sec) a 50 msec value of 2 08 m/sec is found

DISCUSSION

Changes in the *propagation velocity* of an action potential arriving within the recovery phase of a preceding one have been studied in different excitable tissues by many authors Gotch in 1910 reported a longer stimulus to response interval for the second impulse than for the first in nerve and proposed that conduction velocity was retarded during the recovery after a previous im pulse At low temperature also a "stimulation fatigue" was assumed to contribute to the extra delay Lucas (1909 1911) made the same findings in muscle but objected theoretically to Gotch s explanation and concluded that the extra delay depended on other factors unknown at the time than decrea sed conduction velocity He introduced the term *Irresponsive Period* for the shortest interval between two electric responses The experiments of Gasser & Erlanger (1925) in frog s sciatic nerve and in the phrenic nerve of the dog supported the explanation offered by Gotch

In 1912 Adrian & Lucas reported a *supernormality in excitability* during the recovery phase in frog nerve In 1920 Adrian showed that the super normal phase was increased and prolonged with increasing hydrogen ion

concentration Gasser & Erlanger in 1930 found that the supernormality in nerve varied with the after potential They showed that supernormality of excitability was purely a phenomenon of threshold not of the amplitude of the action potential which decreased during recovery and reached normal height at the end of the refractory period

Graham (1934) found this close relationship between the after potential and supernormality in excitability and also a *supernormal conduction velocity* in frog nerve Pillat & Heistracher (1960) found a supernormal phase of velocity in papillary muscle and Farmer Buchthal & Rosenfalck (1960) made the same observation in human skeletal muscle However, the entire recovery curve until normal membrane conditions were restored was not investigated and the duration of the supernormal phase has not been determined Buchthal & Engbæck (1963) studied the supernormal phase of excitability and of conduction velocity in frog sartorius muscle at different temperatures At 25°C the velocity of an action potential elicited by a test impulse was found to be increased by about 10 % at an interval of 2–3 times the absolute refractory period (the absolute refractory period 2 1 msec) The total duration of the supernormal phase was not determined Maximal interval between the con ditioning and test pulses was 8 times the absolute refractory period The amplitude of the action potentials was never supernormal during the recovery phase

The duration of the intracellular recorded negative after potential for the myelinated nerve is 12–20 msec and for the non myelinated nerve 50–80 msec (Grundfest 1940) The time constant of the decay of the after potential in striated frog muscle is about 20 msec (Persson, 1963)

Freygang Goldstein & Hellam (1964) showed the existence of a *late* negative after potential in frog muscle fiber that cumulated to a mean of 10 6 mV during the first 100 msec of stimulation After the stimulation it decayed with a time constant of about 300 msec

One explanation of the negative after-potential in squid axons was offered by Frankenhauser & Hodgkin (1956) who described a small space about 300 A thick between the excitable membrane and the external solution where effluxing potassium ions were accumulated during the depolarisation due to a net outward flux of potassium which then relatively slowly disappeared into the fiber or into the extracellular compartment A similar intermediate region has also been proposed for frog muscle (Hodgkin & Horowicz 1960) Its volume was estimated to be 1/500–1/200 of that of the fiber Freygang Goldstein Hellam & Peachey (1964) considered this intermediate space to be the transverse tubular system of the muscle fiber Here potassium ions were accumulated and depolarized the membrane the late negative after potential The negative after potential in frog muscle

fibers has been further investigated by Persson (1963) who however con cluded that most of the properties of the negative after potential might be explained by permeability changes in the membrane and not by accumulation of ions in an intermediate region outside the membrane

In most of the experiments presented above somewhat unphysiological conditions had to be used e g unperfused isolated nerve, stretched muscle fiber (to avoid contractions) or decreased temperature (to slow down the conduction velocity and in this way make time measurements possible) Graham & Lorente de Nó (1938) however, studied the recovery phase of blood perfused rabbit and cat sciatic nerve to obtain more physiological conditions Contrary to the earlier findings of Graham (1934 1935) no supernormal velocity was found in these experiments and no satisfactory explanation for the divergences could be offered A supernormal excitability phase was, however, found at least after repetitive stimulation

It is thus still an open question whether the supernormal propagation velocity is caused by a disturbance in ion balance or by changes in the membrane properties during the recovery period or by other factors

Any technical faults that might have caused the velocity interval-dependence can at once be excluded (page 31)

Might changes in the fiber diameter (the contraction wave) cause the variation in propagation velocity? This does not seem very likely since the ame type of VRF as found in this investigation has been obtained in experi ments with stretched fibers in which no contraction wave is present (Buchthal & Engbæck 1963) Also in nerves a similar VRF was reported (see above) Thus the changes in propagation velocity in the recovery phase seems not to be due to changes in fiber diameter during activity

In the experiments where action potentials from voluntarily activated muscle fibers were recorded with two separate needles it was never possible to record from the same fiber with the two needles Therefore a direct study of the VRF over a long distance along the fiber was impossible Some con-clusions from the results can nevertheless be drawn

The latency between the potentials from the two electrodes is equal to the difference in propagation time from the common branching point in the nerve to the electrode Each of these propagation times might vary mainly because of a) variation in the propagation time in the nerve b) variation in the neuro muscular transmission time and c) variation in the propagation time in the muscle fiber (page 58)

When the two fibers were recorded with two electrodes separated by many millimeters the action potentials travelled different distances along the muscle fibers A supposed variation in propagation time in the muscle fiber ought to contribute to the variability in the total latency

In the experiments where the distance between the electrode needles was about 20 mm, the variability in latency was 50–70 μsec where the inter electrode distance was about 10 mm the variability was 25–39 μsec and in the experiments reported by Ekstedt (1964) where the distance between the recording electrodes was very small (about 200 μ) the variability (the jitter) was 10–30 μsec Even here the action potentials might have travelled different distances along the muscle fiber (depending on the distance between the motor end plates) Part of the jitter might therefore still be ascribed to the variability in propagation time in the muscle fiber

The finding that the jitter was considerably greater when the interelectrode distance was increased seems to exclude the possibility that the VRF is a local phenomenon over the Multielectrode

The results from this investigation indicate that the same type of changes in the recovery phase as found in other excitable tissues is also present in the human muscle fiber *in situ*

PROPAGATION VELOCITY DURING ISCHEMIA

SUMMARY

The effect of ischemia on propagation velocity and on the velocity recovery function was studied

1 The extensor digitorum communis muscle was made ischemic by means of a blood pressure cuff with or without previous application of an Esmarch roller

2 During ischemia the velocity could decrease (5 out of 23 cases) increase (3 out of 23 cases) or be unchanged (15 out of 23 cases) in relation to the pre ischemic slope of the velocity curve

3 The velocity recovery function was unchanged in 4 out of 20 cases It lost the supernormal phase in 10 out of 20 cases and in 6 out of 20 cases the supernormal phase changed to a subnormal

4 The influence of the innervation frequency upon the propagation velocity became continuously more pronounced during the period of ischemia

INTRODUCTION

The effect of ischemia on propagation velocity can obviously not be investigated on isolated muscle fibers because these are already deprived of their circulation and oxygenated by perfusion The influence of pure anoxia on the muscle fiber has been studied earlier but the combined effect of anoxia and arrested blood circulation (with prevention of normal removal of metabolites and ions) on the propagation velocity seems not to have been investigated

The present chapter is aimed at elucidating the effect of arrested blood circulation on the mean propagation velocity and on the Velocity Recovery Function (VRF) in the muscle fiber

METHODS

The investigation was performed on 9 healthy men 25–35 years old in the extensor digitorum communis muscle This muscle is fairly easy to keep slightly contracted for a long time It is made ischemic more easily than e g the brachial biceps muscle

The experiments were performed in one of the following ways

A In most of the experiments a blood pressure cuff was placed loosely around the upper arm The muscle was voluntarily activated by lifting a finger and the Multielectrode was inserted During the first 10–15 minutes 10–100 readings

of the propagation time of consecutive action potentials were made every 10 seconds to define the pre ischemic slope of the decrease in velocity For the study of the Velocity Recovery Function (VRF Chapter 6) 1–5 records with 200–400 consecutive readings each were made when the innervation frequency was made very irregular The interspike intervals ranged approximately from 50 to 500 msec

The cuff was then insufflated to 200–250 mm Hg and the subject was asked to keep the same innervation frequency as before ischemia. During the ischemic period the propagation velocity was recorded as before Now and then the innervation frequency was made irregular for the study of the VRF

The circulation was restored after maximally 40 minutes sometimes earlier due to the inability of the subject to activate his muscle The desufflation was made slowly over a period of half a minute because otherwise it was impossible to keep the Multielectrode in position Despite this precaution the fiber was usually lost In 10 experiments the same fiber was still recorded after the desufflation of the cuff In the cases where the recording from a fiber was finished a new fiber was sought and the recording was commenced as soon as possible in order to follow the changes in mean velocity in the postanoxic period

B In some experiments the muscle was activated by electrical stimulation and the propagation velocity measured over the Multielectrode The latency between the stimulating pulse and the action potential was also recorded

C In 3 experiments bloodlessness was aimed at The ischemia was induced by winding an Esmarch roller from the fingers to the axilla The blood pressure cuff was applied and insufflated the roller was removed and the needle was inserted and the recordings made according to one of the above described ways With this method the same fiber could not be investigated before and after the application of ischemia The measurements and other methodological details were the same as those described in Chapter 1

In 2 of the experiments the intramuscular temperature was measured The same technique as described in Chapter 1 was used

RESULTS

General findings

A few minutes after the ischemia was applied the subject felt paraesthesia for some minutes beginning peripherally and reaching the wrist The subjects normally felt increasing pain after 10–15 minutes In some cases a sensation of cold was reported after 10 minutes of ischemia In all experiments the subjects noticed a muscular weakness which was often accompanied by an involuntary compensatory increase in the innervation frequency

During the first few minutes following the restoration of circulation the subject felt the same type of paraesthesia as during the induction of ischemia usually more pronounced the longer the period of ischemia During the first minutes in the post ischemic period spontaneous multiple firing was often recorded The action potentials were generated from one fiber and appeared in bursts with 2–6 potentials in each The time interval between

each discharge was 8–20 msec and they seemed to be triggered by voluntarily activated action potentials The types of activity were the same as found during manifest and latent tetania (see i a Isch, 1963) In some cases fasciculations were seen and in one subject there was a cramp in the forearm for approximately one minute The reactive hyperemia was accompanied by a sensation of warmth

These general findings were in accordance with those of Kugelberg (1944)

Propagation velocity

The effect of ischemia upon the mean propagation velocity varied considerably between the different experiments In 3 cases however, where the same fiber was recorded during two or more periods of ischemia separated by a period of 10–15 minutes with restored circulation essentially the same type of change in velocity was found during the different ischemic periods (Fig 33)

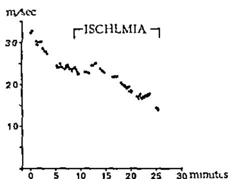


Fig 30 *The effect of ischemia on propagation velocity* During ischemia the velocity seems to decrease in relation to the expected course of the curve

In 5 out of 23 periods of ischemia the slope of the velocity decrease curve became steeper after some minutes of ischemia (Fig 30) In 4 out of 23 experiments there was a tendency to an increase in velocity during the first minutes of ischemia (as seen in Fig 31)

In 15 out of the 23 experiments no significant change could be detected between the slopes of the pre ischemic and ischemic curves (Fig 32) In 3 of the experiments the velocity increased during the whole period (10 10 and 12 minutes) of ischemia (Fig 33)

Usually the mean innervation frequency was about 10 impulses/sec As reported in Chapter 4, the propagation velocity decrease curve became steeper when the innervation frequency was increased The influence of innervation frequency upon propagation velocity became continuously more pronounced during the period of ischemia

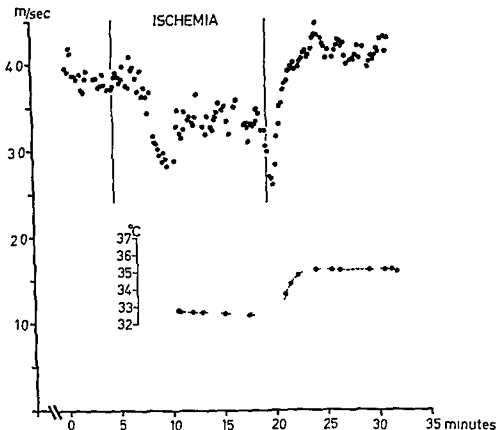


Fig 31 *Influence of a period of ischemia on propagation velocity* The velocity is shown to decrease during the ischemic period Immediately after restoration of circulation the intramuscular temperature rises and propagation velocity increases Each value of propagation velocity is the mean value for all action potentials occuring during a 10-second period

In the 14 experiments where it was possible to measure the propagation velocity directly after the restoration of the circulation a transitory increase in mean velocity was seen in 3 cases The increases were 30 % 31 % and 35 % above the value at the end of the ischemic period and reached a maximum after 4–5 minutes (Fig 31) The velocity then decreased again

The Velocity Recovery Function

The propagation velocity values were treated in one of two ways according to the principles given on page 64 for the study of the VRF the velocity values were plotted against continuous time or the changes in propagation velocity from the preceding impulse was plotted against interval (Figs 34 and 35)

FIG 32 *A continuous recording of propagation velocity during 3 hours and 17 minutes* Each value is the mean value of at least half – usually all – the propagation velocity measurements during 10 seconds Ischemia has been applied during four periods (I) in all of which the velocity decreases

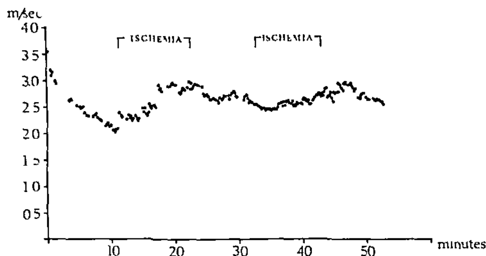


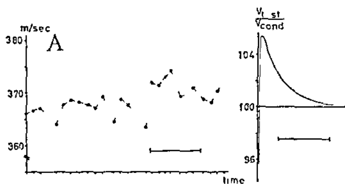
FIG 33 *The effect of ischemia on propagation velocity* The propagation velocity increases during both the ischemic periods

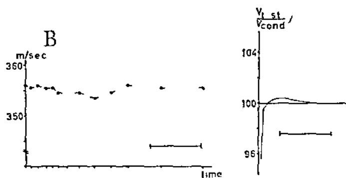
In the pre ischemic p_riod the VRF was of the general form as described on page 63 in all of 20 fibers In 4 of these cases there was no gross change of the supernormal part during 7 12 19 and 30 minutes of ischemia In 10 cases the supernormal part disappeared after 3 5, 6, 6 8 10 10, 13 15 and 20 minutes of ischemia but no subnormality was seen In 6 cases however the VRF was changed from the normal appearance to that shown in Fig 35 with a subnormal phase after 4 8 9 13 13 and 25 minutes Ten minutes after restoration of the circulation the VRF seemed to be normalized in 2 out of 3 cas_s To clarify the magnitud_ of the changes in VRF some of the results are presented in Table 4

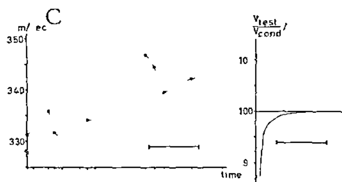
TABLE 4

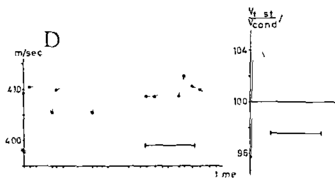
Changes in the parameters of the V-locity Recovery Function during ischemia

Experiment Number	Tim Minutes	Notation	Calculated V_{basal} m/sec	Calculated propagation velocity increase at 50 msec for the basal class (in percent)
1016	0		2 4	4 7
	2		2 1	4 2
	4		2 0	5 2
	5		1 9	8 2
	7	Start of ischemia		
	8		2 1	4 8
	9		2 3	3 4
	12		2 3	2 3
	18	End of ischemia		
	19		2 6	1 6
	22		2 4	6 2
	23		2 4	4 9
	24		2 3	3 6
	29	Start of ischemia		
	30		2 3	5.5
	31		2 3	6 2
	33		2 2	6 9
	36		2 3	6 2
		End of ischemia		
1044	0		2 6	4 7
	1		2 5	4 7
	2		2 5	4 6
	3		2 4	4 6
	4		2 3	2 4
	10	Start of ischemia		
	12		2 1	-1 0
	14		2 2	-1 2
		End of ischemia		


A
m/sec
380
370
360
time
$\frac{V_{test}}{V_{cond}}$
104
100
96


B
m/sec
360
350
time
$\frac{V_{test}}{V_{cond}}$
104
100
96


C
m/sec
350
340
330
time
$\frac{V_{test}}{V_{cond}}$
100


D
m/sec
410
400
time
$\frac{V_{test}}{V_{cond}}$
104
100
96

Temperature

During 2 of the experiments the temperature was also recorded During the ischemia the intramuscular temperature decreased 1 °C during 20 minutes in one case 0 5 °C during 30 minutes in another After the relief of ischemia the temperature increased 1 5 and 2 6 °C respectively during the first 5 minutes, and was thus above the pre ischemic level After another 5–10 minutes it slowly began to return to the initial value

DISCUSSION

The main findings concerning the propagation velocity during ischemia were The slope of the velocity decrease curve was usually steeper than normal but sometimes no change was detectable In 3 cases there was even a slight increase in velocity

A transitional increase in velocity was sometimes seen when the ischemia was applied and when the circulation was restored

For a given change in the mean innervation frequency the propagation velocity changed more at the end of the period of ischemia than at the beginning

The Velocity Recovery Function (VRF) usually changed heavily in shape during ischemia and returned to its normal appearance after a short time when the circulation was restored

Due to the variations in the obtained results no general conclusion concerning the effect of ischemia on propagation velocity could be drawn Since in experiments without ischemia occasional variations in the velocity of the same order of magnitude are found as during ischemia even rather obvious changes concomitant with the ischemic period have to be analysed in relation to normal variations Sometimes ischemia seemed to induce a somewhat steeper slope of the velocity decrease curve The reason for this effect might be that the factors normally causing the velocity decrease are strengthened or that new factors are added If the normal decrease in propagation velocity

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Fig 34 *Influence of ischemia on the velocity interval-dependence* *A* Diagram to the left is Velocity Time Diagram i e the variation of propagation velocity (ordinate) plotted against continuous time (abscissa calibration line 1 sec) To the right is illustrated a possible form of the Velocity Recovery Function not calculated Ordinate Propagation velocity of the testing action potential (V_{test}) in relation to the conditioning (V_{cond}) in per cent Abscissa Interval between conditioning and testing action potential Data from the pre ischemic period *B* After 10 minutes of ischemia the supernormal phase of the VRF is diminished *C* After 14 minutes of ischemia the VRF is inverted and is subnormal in its whole course *D* 4 minutes after restoration of circulation the VRF is normalized

were related to an accumulation of metabolites restricted circulation might have the effect seen in the present experiments

The reason for the great variability in effect of ischemia upon the velocity might be either a) different inherent characteristics in the fiber or b) different conditions for the fibers in the different experiments

a) Different fibers might have a different energy consumption during activity which might explain the varying effect of ischemia upon them Another possibility is that a greater membrane surface of a fiber makes it more sensitive to disturbances in normal circulation The present data did not allow any statistical analysis of the correlation between the propagation velocity (as an arbitrary measure of the fiber diameter see page 98) and the amount of decrease during ischemia However the experiments where the propagation velocity of one and the same fiber responded to ischemia in the same manner during different periods of ischemia, indicate that inherent factors are responsible for most of the observed differences between the fibers

b) If the region around the fiber under study had been badly perfused (because of the normal variations of local circulation in the muscle) during the time preceding the ischemia this might have strengthened the effect

During periods of ischemia there was in many experiments a tendency to an increase of the mean innervation frequency when the subject was not repeatedly ordered to keep it constant In the present experiments however the mean frequency was kept constant within approximately the normal range of variations during the whole experiment Furthermore there was no essential difference in the curves obtained with electrical stimulation and voluntary activation and therefore the change in velocity during ischemia cannot be ascribed to changes in innervation frequency

The increase in propagation velocity during the first minutes following the application of ischemia and during the first minutes after restoration of ischemia might be of the same nature as described for nerve by Kugelberg (1944) Magladery MacDouglas & Stoll (1950) and Gilliatt & Willison (1963) These authors considered the increase in velocity to be due to a lowered threshold of the membrane

The consistent finding of an increasing influence of the innervation frequency on the propagation velocity during ischemia has also been reported by Bullock (1951) This might be due to a superimposed effect on the decrease in basal velocity of an inverted VRF When the VRF has a subnormal part in the time interval of 50–500 msec (the limits for the intervals usually appearing in the voluntarily activated muscle Fig 35) an increase

in innervation frequency will decrease the mean velocity and accentuate the change in velocity

The change in VRF during ischemia was a fairly constant finding In cases where the ischemic period was longer than 15 minutes there were obvious changes in the shape of the VRF (Fig 35) Corresponding changes were not seen during continuous activity for more than 30 minutes without ischemia When the supernormal part changed to a subnormal for the actual interspike intervals (50–500 msec), a stage was passed in which the curve was flat and the propagation velocity fairly constant for consecutive discharges even at irregular frequencies In some cases, however, the curve seemed to pass through some more complex form not exactly known before the subnormal part was seen During this intermediate stage no correlation was found between interval and change in velocity

The change in VRF during ischemia might be responsible for at least part of the change in mean velocity in the following way A mean innervation frequency of 10/sec (which is a common value in the experiments) was assumed With a normal VRF the mean velocity would be increased by about 5 % above the basal level With the VRF inverted by ischemia (Fig 35) the velocity would be decreased by about 5 % below the basal level at the same innervation frequency The continuous change of the VRF during an experiment might thus change the velocity from 105 % to 95 % of the basal i e a decrease by about 10 %

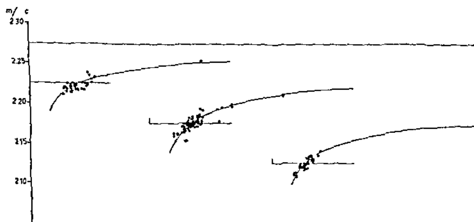


Fig 35 *Inverted Velocity Recovery Function after 9 minutes of ischemia* For explanation of the principles for drawing of the figure see fig 27 The horizontal line in the upper part of the figure is the asymptotic line which the three curves are approaching

The increase in velocity, seen in the early postanoxic period might be ascribed to the normalisation of the VRF, but still other factors could theoretically be responsible for this effect One such factor is the increased temperature that was actually observed in the experiments Another factor is an increase in excitability such as has been reported to occur in nerve (page 86)

The fact that it was impossible in most cases to record from the same fiber before during and after the period of ischemia makes the results somewhat more difficult to interpret The main conclusions, however concerning the propagation velocity and the VRF seem not to be affected by this fact Ischemia seems to influence the mean propagation velocity to only a minor degree much less than the effects of changes in innervation frequency The VRF is, however entirely changed in most of the fibers

Actual measurements of propagation velocity in muscle fibers during ischemia have not appeared in the literature However, the findings presented above are in general agreement with what was known earlier about the effects of anoxia

Farmer Buchthal & Rosenfalck (1960) reported a change in the recovery cycle in human muscle fibers during ischemia The refractory period was prolonged from 2 2–4 6 msec to 5 0–6 2 msec

In isolated muscle fibers from the frog the after depolarisation (negative after potential) has been shown to be more sensitive to change in the electro lyte milieu (Macfarlane 1952) and in metabolic activity (Macfarlane & Meares 1955 1958*a*) than the spike and the resting potential In experiments with anoxia Macfarlane & Meares (1958*a*) found that the after potential in frog muscle fibers was reduced within 20 minutes at which time the action potential was still mainly unchanged The after potential recovered within 10 minutes of the return of oxygen

The effect of anoxia upon the resting and action potentials and after potentials in nerve fibers has been investigated by many authors (for references see Macfarlane & Meares, 1958*a*) Essentially the same results as those obtained with muscle fibers were reported The propagation velocity in whole nerve *in situ* was measured by Gilliatt & Willison (1963) who found an increase of 12 % during the first 3–5 minutes of ischemia and during the same period an initial increase of excitability, followed by a decrease

The fact that a change occurs in a muscle fiber parameter (the VRF) under ischemic conditions might prove of value in the further study of muscular disorders and the effect of some pharmacological agents on muscle fibers

PROPAGATION VELOCITY AT DIFFERENT TEMPERATURES

SUMMARY

1 The intramuscular temperature of the brachial biceps muscle was changed between 22 1 and 39 0°C by alteration of the external temp rature of the upper arm

2 In relation to the normal change in propagation velocity during activity an increase in temperature up to 39°C seemed to increase the propagation velocity in all of 6 cases When the temperature was decreased by 9°C significant changes were seen in 2 out of 5 cases When the temperature was decreased by 4°C in 3 cases no change was seen

3 An increase in temperature from normal did not alter the Velocity Recovery Function in the one case where this was studied A decrease of the normal temperature below 30 C made the supernormal phase disappear in the 3 cases where this was studied

4 The variation in intramuscular temperature when the environmental conditions were reasonably constant were small and does not appreciably influence the propagation velocity or the Velocity Recovery Function

INTRODUCTION

Only a few reports are found in the literature concerning the propagation velocity and temperature Wilska & Varjoranta (1940) found a nearly linear relationship b tween temperature and propagation velocity in the muscle fibers of the abdominal muscle of the frog When the temperature was in creased from 0 to 36°C the velocity increased more than five times In isolated frog muscle in whole muscle preparation Buchthal & Engbæck (1963) investigated the velocity in the temp rature range of 14–25°C and found a positive correlation with a $Q_{10} = 1\ 6$ In intact human muscle Buchthal & Rosenfalck (unpublished data cited by Buchthal & Sten Knudsen 1959) found a temperature coefficient (Q_{10}) of 2 in the range of 30–37°C Between 26 and 30°C there was no significant change in propagation velocity with temperature In human nerve the velocity decreased at decreasing temperature (see Gassel & Trojaborg 1964)

The propagation velocity during the recovery phase following an action potential has not been studied with regard to its dependence on the temperature in muscle fibers In nerve Tasaki (1949) found that the supernormal

velocity in the recovery phase disapperared and that the refractory period increased at cooling

The experiments presented in this chapter were made in order to evaluate the effect of temperature upon the propagation velocity and on the Velocity Recovery Function

METHODS

The experiments were performed in the brachial biceps muscle in 4 healthy young men The muscle was voluntarily activated with a slight contraction The general methods for recording and for the measurements of the temperature is described in Chapter 1 The thermo needle was placed about 1 cm from the Multielectrode at the same depth from the surface

The experiments did not begin until several minutes after the insertion of the Multielectrode and thermo needle During this time the temperature differences between the needles and the surrounding tissue were equilibrated

The temperature was changed in the muscle by means of the following device A 1 5 mm thick rubber tube 1 cm in diameter loosely twisted round the upper arm about 20 turns The tube was connected to a water tap with warm or cold water The Multielectrode and the thermo needle were inserted between two turns of the tube care being taken that there was no contact between needles and tube In this way the temperature in the biceps muscle could be changed from 39°C (pain threshold) to 22 1°C (below which temperature no action potentials could be obtained)

RESULTS

General findings

Spontaneous temperature changes When the thermo needle was placed in different positions in the muscle and there was no water in the tube the temperature was found to vary within the muscle by about 2°C The lowest values were obtained near the surface

Some minutes after normal activity had ceased the temperature slowly decreased At slight contraction however, the temperature was constant within 0 8°C for a time period of 30 minutes

Artificially changed temperature When the temperature in the tube was changed the intramuscular temperature in the vicinity of the thermo needle began to change within one minute The temperature increased approximately 0 5–1 6°C per minute and decreased 0 2–0 8°C per minute within the temperature range of 28–37°C

At *low temperature* below 30°C the subjects often had a feeling of weakness and when the temperature was decreased below 27°C occasional drop out of potentials was noticed (a single action potential in a sequence of otherwise regular discharges would be lacking) The total duration and the rise time of the action potentials increased in gross proportion to the

decrease in velocity The amplitude of the action potentials was usually lower than normal In one experiment the temperature was 22 1°C Here no activity could be elicited with voluntary contraction but with electrical stimulation action potentials with low amplitude and a slow rise time were recorded

At *high temperature* (39°C) no gross change in the potential form was seen The innervation frequency and rhythm was normal

Propagation velocity

Decreased temperature Activity from about 20 fibers were recorded but as the experiments had to go on for more than 10–15 minutes recordings from only 5 fibers could be used for analysis The other fibers were lost because of some of the factors described on page 42 or because of difficulties in activating the muscle fibers when the temperature was low In 2 out of 5 cases the velocity decrease curve became significantly steeper when the temperature was decreased about 9°C (from 39°C to 30°C and from

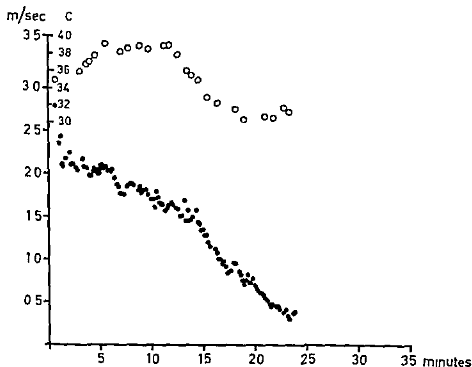


Fig 36 *The effect of temperature on propagation velocity* Abscissa continuous time Ordinate intramuscular temperature (upper) and propagation velocity Note the time lag between the change in temperature and change in propagation velocity

Increased temperature In one experiment the temperature had been kept at 29°C for 12 minutes during which time the VRF had lost the super normal phase After this period the temp rature was raised to 35°C within 7 minutes The VRF was continuously normalized and after 5 minutes at about 34°C there seemed to be a normal velocity interval dependence When the temperature in one experiment was 39°C for 10 minutes no gross changes in the VRI from normal were found

DISCUSSION

Although the material is small it seems to be possible to draw some conclusions from these experiments concerning the effect of temperature upon the electrical activity of the muscle fiber The propagation velocity and the VRF in all the investigated fibers were influenced by changes in tempe rature in a reproducible way, but no quantitative analysis has been mad

There was a positive relationship between temperature and the propa gation velocity in the temperature interval of 23–39°C Below 23°C no action potentials could normally be elicited and 39°C was the maximal intramuscular temperature obtained with this technique before the subjects felt too much pain

When the intramuscular temperature was made to increase after a period of continuous decrease or the reverse there was usually a certain delay of 1–10 minutes before the propagation velocity responded to the change This delay could not be ascribed to error in measurements of tempe rature i e different depths of the thermo-electrode and the Multielectrode (the same delay was seen when the thermo electrode was inserted 0 5 cm deeper than the Multielectrode as when it was 0 5 cm nearer the surface) The cause of this delay is not known

The cause of the dependence of propagation velocity on temperature was not further studied Experimental errors such as change in mean innervation frequency or ischemia due to pressure of the water tube can be excluded The delay mentioned above would seem to indicate that metabolic factors are involved

Since ion movements are involved in bioelectrical phenomena it seems reasonable that a lower temperature slows down the rate of these processes The effect on the propagation velocity is however much more complex shown for example by Macfarlane & Meares (1958*b*) They showed that the rate of rise of the intracellular action potential (in frog sartorious muscle) increased with temperature but that the voltage of the action potential decreased The same findings have been made by Hodgkin & Katz (1949 *b*) in the giant axon of the squid These two changes influence the propagation

velocity in opposite directions (Hodgkin & Katz 1949*a*) Another example showing the complex influence of temperature is given by Fitzhugh (1966) in a theoretical analysis of the Hodgin Huxley (1952) nerve model He found the stimulation threshold temperature curve to be U shaped for short stimulating pulses but monotonically increasing for long pulses

The effect of different temperatures upon the VRF with a decrease in the supernormal phase at cooling is in accordance with what was found in the motor nerve of the toad by Tasaki (1949) He found that the recovery processes slowed down and that the supernormal phase disappeared between 12°C and 6°C The change of the recovery phases in the muscle fiber has not been studied earlier The negative after potential however, which is correlated to the supernormality in velocity (page 75) was found to decrease at low temperatures (Macfarlane & Meares 1955 1958) in frog sartorious muscle and to increase in toad sartorious muscle with increasing temperature (Ishiko & Sato, 1957) Corresponding figures are reported for nerve (Lorente de No 1947)

From the practical point of view the conclusions can be drawn that the intramuscular temperature variations at constant environmental tempe rature are small and cannot appreciably alter the propagation velocity and Velocity Recovery Function

PROPAGATION VELOCITY IN DIFFERENT MUSCLES

SUMMARY

1 The propagation velocity was measured in different human muscles in situ

2 The mean velocity in the voluntarily activated brachial biceps muscle in twelve healthy subjects was 3 69±0 71 m/sec (n=443) The same value was found with electrical stimulation

3 There was a positive relationship between the circumference of the upper arm and the mean propagation velocity in the brachial biceps muscle

4 There were differences in mean propagation velocity in different muscles e g 2 01±0 39 m/sec (n=26) for the frontal muscle 3 15±0 75 m/sec (n=147) for the extensor digitorum communis muscle and 3 39±0 68 m/sec (n=142) for the femoral quadriceps muscle

5 In one case of disuse atrophia of one of the femoral quadriceps muscles the mean propagation velocity was significantly lower in this muscle than in the normal

INTRODUCTION

Earlier experiments indicate (page 11) that there exists a correlation between propagation velocity and fiber diameter both in isolated nerve and muscle fibers In human muscle only a few measurements of propagation velocity have been made *in situ* Only Buchthal Guld & Rosenfalck (1955*b*) have analysed the velocity diameter relationship and proposed that for the fiber *in situ* the diameter influences propagation velocity only to a minor degree

In this chapter propagation velocity (measured within a defined time interval after the insertion of the needle) of a number of muscle fibers in different subjects and in different muscles in the same subject was measured An attempt was made to correlate the propagation velocity with the fiber diameter but this study was not aimed at finding out the mathematical equation for the relation between these parameters, but at getting a preliminary view of propagation velocity values in different fibers and to compare different muscles Some experiments on pathological muscles were also performed

METHODS

This study was made at an early stage of the investigation and the wobbler technique (page 17) was used in most of the experiments The *3+11* Multi electrode was used

The muscles were voluntarily activated at the same time as the needle was inserted After the necessary corrections of the needle position usually finished within 30 seconds the propagation velocity was recorded for many minutes The mean value of 5–15 measurements during the 3 minutes following the initial 30 seconds was taken as a measure of the propagation velocity of the muscle fiber A new needle position was sought for each fiber recorded

In experiments on normal muscles 443 fibers were measured in 12 normal brachial biceps muscles 147 fibers in 7 extensor digitorum communis muscles 26 fibers in two frontal muscles 142 fibers in two femoral quadriceps muscles In other experiments 49 fibers in brachial biceps muscles from 2 children were studied

One atrophied and one trained femoral quadriceps muscle in one patient were examined (72 and 42 fibers respectively)

In one series of experiments 92 fibers in 2 brachial biceps muscles were electrically stimulated (2–5 impulses/sec) and the propagation velocity measured with the Multielectrode technique

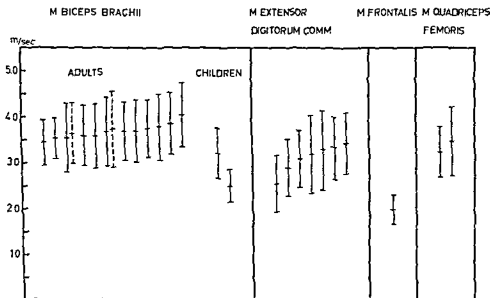


Fig. 39 *Propagation velocity in different muscles* Abscissa different samples in the different muscles arranged in ascending order of propagation velocity Ordinate propagation velocity ±1 SD The samples which are marked with dashes have been obtained with electrical stimulation from the respective subject

RESULTS

The mean propagation velocity in samples of muscle fibers in the brachial biceps muscles in healthy subjects varied from 2 48±0 34 m/sec in a

child (with a circumference of the upper arm of 16 6 cm) to 4 04±0 70 m/sec in a well trained subject (circumference 32 6 cm) The mean value for the whole material (adults) was for the brachial biceps muscle 3 69±0 71 m/sec (n=443) for the extensor digitorum communis muscle 3 15±0 75 m/sec (n=147) for the femoral quadriceps muscle 3 39±0 68 m/sec (n=142) for the frontal muscle 2 01±0 39 m/sec (n=26) The mean velocity in the brachial biceps of two children, 4½ and 6½ years old was 2 48±0 34 m/sec (n=10) and 3 23±0 56 m/sec (n=39) respectively In both cases the differences from the normal material is highly significant ($p < 0\ 01$) See Fig 39

As to the subjects where the circumference was measured (7 cases) there was a positive correlation (Fig 40) between the circumference of the upper arm and the mean velocity in the muscle ($p < 0\ 05$)

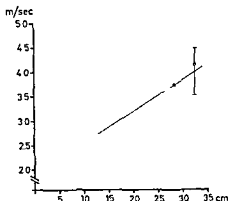


FIG 40 *Propagation velocity in the brachial biceps muscle plotted against upper arm circumference* The regression line calculated with the method of least squares (°) indicates the mean value for a sample in a case of acromegalia (not used in calculation of the regression line) The line through that value indicates the standard deviation for the normal material at this circumference

The results of the measurements with the Multielectrode after electrical stimulation were within the range of the values obtained from the voluntarily activated normal muscles

One patient had his right knee joint immobilized for 3 weeks in a plaster cast after an operation upon a meniscus (Fig 41) One month after removal of the plaster the first measurements were made in his quadriceps muscles In the normal leg (circumference 43 5 cm 13 cm above the patella) there was a mean velocity of 3 48±0 74 m/sec (n=82) in his operated leg (circumference of 39 5 cm) the mean velocity was 2 78±0 36 m/sec (n=38) The difference between the mean values is significant ($p < 0\ 01$) and also th difference in standard deviation (variance ratio 4 2 $p < 0\ 01$ Moroney 1960) One year later the mean velocity in the normal leg (circumference of 42 3 cm) was 4 23±0 55 m/sec (n=42) and in the quadriceps muscle formely immobilized (circumference of 40 8 cm) the velocity was

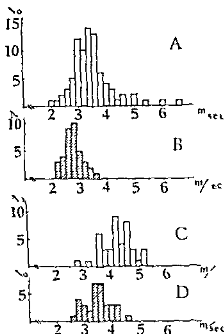


FIG 41 *Effect of disuse atrophy on propagation velocity* See page 98 Frequency distribution of samples of propagation velocity in both quadriceps femoris muscles

One month after removal of the plaster cast
A Normal left leg
B Atrophied right leg

One year later
C Normal left leg
D Atrophied right leg

3 49±0 48 m/sec (n=34) The difference between the mean values is significant ($p < 0\ 01$) but not the difference in standard deviation

DISCUSSION

It is known from literature (cf page 11) that there is a correlation between the diameter of the isolated muscle fiber and its conduction velocity in vitro For human muscle fibers in situ however Buchthal Guld & Rosenfalck (1955 *a b*) found the velocity in voluntarily activated muscle to be 4 72±0 54 m/sec (n=26) and for electrically activated fibers 4 02±0 43 m/sec (n=16) despite the fact that fibers were known to range from 25–85 μ in diameter in the normal biceps muscle This small variability in velocity was ascribed to a high external resistance due to the arrangement of the fiber in subunits The fiber under study and those around it were all considered to be activated synchronously and from the point of view of the actual fiber the external resistance seemed to be high acting to minimize the influence of diameter

In the experiments described in this chapter it was generally found that mean values of propagation velocity during the period 30–210 seconds after the insertion of the needle ranged from for example 1 5 to 5 8 m/sec in the same muscle (mean 3 67±0 75 m/sec n=159) The mean values

were less in small muscles (the frontal muscles) than in coarse (the femoral quadriceps and brachial biceps muscles) The found correlation between the circumference of the upper arm and propagation velocity in the biceps muscles might indicate a relationship between fiber diameter and propagation velocity

The mean velocity as defined in this chapter was about 13 % lower than it would have been if the velocity had been measured at the time for insertion of the needle (Table 1) As the decrease in velocity (in m/sec) did not seem to be correlated to the initial velocity value, the range of measured velocity was mainly the same as if the velocity had been measured at the time for insertion of the needle

Another question that arises when measuring a sample of muscle fibers with this method is whether there is any overrepresentation of potentials from large fibers because of their higher amplitudes or of small fibers because there are more small fibers than large ones per unit muscle area To little is known about the volume conduction of the currents in the muscle to answer this question Is there any biological bias in these experiments when the muscle is only slightly activated? It is known that smaller nerve fibers have a higher electrical threshold than larger ones but no reports are found in literature as to whether there is any difference in mean value of muscle fiber diameter between motor units with large and those with small nerve fibers It is not known which motor units are recruited first at the slightest contraction It must therefore be stressed that the present experiments with voluntarily activated fibers are made on samples of fibers recruited at slight effort

The absolute values of propagation velocity were somewhat lower than at zero time because the fiber had worked for 3 minutes As no difference in this decrease has been detected in different muscles, the comparisons between the mean velocity could be made

The present experiments speak in favour of a positive correlation between velocity and fiber diameter

SOME FINDINGS IN MUSCULAR DISORDERS

SUMMARY

1 The mean value of propagation velocity was lower than normal in one case of disuse atrophia and in two cases of dystrophia myotonica

2 The propagation velocity was within the normal range in 5 cases of myxoedema

3 In one of two cases of acromegalia the mean velocity was significantly higher than in healthy subjects

4 The mean propagation velocity seemed to decrease during thyroid therapy in a case of myxoedema

5 The Velocity Recovery Function was shown to be changed in myxoedema but seemed to be normal in dystrophia myotonica and myasthenia gravis

INTRODUCTION

During the course of the study of propagation velocity in human muscle fibers a few patients with muscular disorders have been investigated to give an idea whether the method might be of value in the study of muscular diseases

METHODS

The same general methods as described in Chapter 1 were used
The *3+11* and *1+13* Multielectrodes were used

RESULTS

Propagation velocity

In a patient already described on page 98 and in Fig 41 with a mild *atrophia* in his quadriceps femoris muscle due to inactivity there were significant differences between the mean values of propagation velocity in samples of muscle fibers from the atrophied and normal leg One year later the circumference of both legs had increased and the propagation velocity in the muscle fibers in both quadriceps had increased as well

In 5 patients with *myxoedema* the propagation velocity values in the exten

sor digitorum communis muscle were within the normal range The values given for adults in Chapter 9 are used as normal values (3 15±0 75 m/sec, n=147) In one of the patients measurements in a sample of muscle fibers in the extensor digitorum communis muscle were made before and 1 and 2 months after beginning of thyroid therapy The mean velocity decreased from 3 73±1 05 m/sec (n=10) to 3 05±0 78 m/sec (n=13) The difference between the mean values is probably not significant 0 05 < p < 0 1) Evidently more fibers have to be investigated

In two patients with *dystrophia myotonica* with an obvious muscular atrophia the mean velocity in the brachial biceps muscle was 2 52±0 72 m/sec (n=11) and 2 42±0 30 m/sec (n=6) which is significantly lower than in healthy subjects (p < 0 01)

Two patients with *acromegalia* were examined One had a significantly higher mean propagation velocity in the brachial biceps muscle than normal 4 15±0 87 m/sec (n=26 p < 0 01) However this mean velocity falls on the linear regression line of the propagation velocity on the circumference of the upper arm in normals One had a mean propagation velocity in brachial biceps within the normal range 3 63±0 70 m/sec (n=16 0 3 < p < 0 4) The circumference was not measured

Velocity Recovery Function

In myxoedema the VRF of 10 of 20 fibers had an abnormally small supernormal phase In 2 cases a subnormal phase was seen (for the intervals 50–500 msec) and this was never seen in healthy subjects In 2 cases however the supernormal phase seemed to be more pronounced than normally seen at an interval of 50 msec the velocity was increased by 30 %

In two cases of *dystrophia myotonica* about 20 fibers and in five cases of *myasthenia gravis* about 15 fibers were investigated The shape of the VRF did not seem to differ from the normal

DISCUSSION

In ordinary electromyography the motor unit activity is the subject of study and the compound action potentials from several fibers are recorded The method used in this investigation however gives information about the single fiber

The finding of a decrease in mean propagation velocity in the muscle with *disuse atrophia* is of interest as this type of atrophia cannot be diagnosed by ordinary EMG

The smaller standard deviation of the propagation velocity values from the atrophied muscle and the fact that the lower limit of the distribution did

not fall below that in the normal leg might indicate that the larger fibers had decreased more in diameter than the smaller ones Further study is needed

The high mean velocity values in the patients with *acromegalia* can probably be ascribed to the large fiber diameter which these patients are known to have

In *myxoedema* there are different muscular symptoms of which the slowed relaxation is most common The muscle fibers are swollen as a result of accumulation of mucinous substances Electromyographic changes in the form of reduction in size and duration of the motor unit potentials and increased proportion of polyphasic potentials are reported (e g Astrom Kugelberg & Muller 1961) The first patients with myxoedema were investigated on the basis of the question whether the propagation velocity was lowered when the basal metabolic rate was low All the 5 patients however were found to have the propagation velocity within normal limits In one patient studied before as well as during therapy the velocity showed a tendency to decrease during treatment The propagation velocity is thus not decreased (because of the low metabolic rate) but might be increased because of the fiber swelling This requires further study

The membrane processes underlying the change in velocity in the recovery phase are dependent on metabolic factors (page 88) The change in the VRF in the patients with *myxoedema* might be an effect of changed metabolic activity in the muscle fiber

The velocity was decreased in the 2 cases of *dystrophia myotonica* Whether or not this decrease can be ascribed to the decrease of the muscle fiber diameter or to other factors as well cannot be settled at present The present analytical treatment for the VRF did not indicate any difference from the normal However, in muscular dystrophies it is known that there are changes in the properties of the muscle fiber membrane (Farmer Buchthal & Rosenfalck 1959 Norris 1962) Therefore the mathematical analysis of the VRF is undergoing further development to make it more sensitive to detect changes that could be present in this disease

GENERAL SUMMARY

The propagation velocity of single muscle fiber action potentials in voluntarily activated human muscle *in situ* has not earlier been studied The present monograph is aimed at developing a method for such measurements and at studying the influence upon the propagation velocity of some factors

In *Chapter 1* the methods are described The method is based on the use of a needle Multielectrode In the side of the needle (0 6 mm in diameter) are mounted 14 platinum electrodes each of diameter 25 μ The electrodes are arranged in a pattern that permits the recording of single muscle fiber action potentials from one and the same fiber with two electrodes at a mutual distance of about 250 μ along the fiber The time lag between action potentials so recorded (of the order of magnitude of 50–200 μsec) is used for calculation of the propagation velocity

Because of the small distances and short time intervals that are measured it is necessary that the needle Multieelctrode be correctly positioned in relation to the fiber to obtain reliable results In *Chapter 2* criteria for correct position are discussed and an analysis made of the errors in measurement The maximal errors in the measurement of the propagation velocity were thus found to be +4 % and —2 % Slow variations could occur during an experiment due to unnoticed position changes giving errors of the order of magnitude of $\pm$1 % The variations in propagation velocity between consecutive measurements that are caused by technical errors can not have exceeded 0 5 %

It would have been valuable to compare the propagation velocity over a longer distance of the muscle fiber with the values obtained over the 250 μ distance in the Multielectrode An attempt was therefore made to stimulate electrically single muscle fibers *in situ* In *Chapter 3* the results of these experiments are presented It was not possible to restrict the stimulation to only one or a few muscle fibers either when using a needle electrode in the human muscle, or when using sharpened tungsten wires in the rabbit muscle The stimulation always gave a response in a great many fibers However, it was possible to obtain a more or less polyphasic potential in which single

muscle fiber action potentials could be discerned and recorded This type of response disappeared after injection of d tubo-curarine in the rabbit and was therefore considered to have been caused by stimulation of nerve twigs One or several motor units must thus have been activated

In *Chapter 4* the results of propagation velocity measurements during long continuous voluntary activity are reported The velocity was always found to decrease from the initial values most pronounced during the first 10 minutes In the brachial biceps muscle (initial mean propagation velocity 3 37 $\pm$ 0 67 m/sec SD) the velocity decreased 1 09 $\pm$ 0 43 m/sec (SD) during the first ten minutes and in the extensor digitorum communis muscle (initial mean propagation velocity 3 34 $\pm$ 0 69 m/sec SD) the decrease was 0 85 $\pm$ 0 37 m/sec (SD) during the first ten minutes There was no difference in rate of decrease between fibers with high or low initial propagation velocities There were also slow variations in propagation velocity up and down The innervation frequency was shown to influence the rate of decrease of propagation velocity at low frequencies the decrease was slower than at high frequencies After a pause in activity the values were higher than immediately before the pause When the Multielectrode was inserted 30–50 minutes before the activity began the velocity decreased similarly to the curves obtained in the normal way

The following question now arose Are the changes in velocity that were shown with the Multielectrode technique representative for the whole muscle fiber, or are they only local changes in the vicinity of the needle caused by the mere presence of the needle? This problem was dealt with in *Chapter 5* Direct stimulation of a muscle fiber could not be made as shown above Attempts were made to record the activity from the same fiber with two needle electrodes but this failed Therefore experiments with stimulation of a nerve fiber and recording from its muscle fiber were made The experiments seemed to indicate that the decrease in propagation velocity over the Multielectrode was mainly but not exclusively a local phenomenon Further investigations are needed

When analysing the propagation velocity measurements it was found that the variability in velocity was greater than could be ascribed to technical errors In *Chapter 6* this variability was studied and was found to be dependent on the time interval between the action potential discharges When the time interval from the preceding action potential was short (below 5–10 msec) the velocity was lower than for the preceding action potential when it was longer the velocity was supernormal with a maximum of up to 25 % at 8–50 msec A method was developed for the analysis of this *Velocity Recovery Function* from the data obtained at voluntary contraction with the

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In the retina high AChE* activity has been demonstrated in the inner plexiform layer and in the ganglion cell layer but no or very low in the outer nuclear layer (for review see Eichner 1958 Esila 1963, Koelle 1963 Rohen 1964) The AChE activity increases during differentiation in vivo of the nerve cells (for discussion see Karczmar 1963 Couteaux 1963) After damage of nerve structures the AChE activity soon reaches its normal level after an initial decrease (for discussion see Fredricsson and Sjoqvist 1962 Harkonen 1964)

Recent electron microscopic and electrophysiological studies on cultured nervous tissue have demonstrated synapses in the explants (Bunge Bunge and Peterson 1964 Crain and Bornstein 1964 Callas and Hild 1964 Crain and Peterson 1964 Peterson Crain and Murray 1965, Murray 1965) Except for the studies of Geiger and Stone (1962) which have recently been criticized (Bornstein 1964 Murray 1965) no other histo chemical study of AChE activity in cultures of nervous tissue has been published

Previous studies on retinal cultures have demonstrated that the nerve cells develop with time in vitro (Hansson and Sourander 1964 Hans son 1965 1966) This study was performed to investigate if AChE occurs in the nerve cells including their processes in vitro As there are many histochemical methods for AChE and no reliable studies on AChE activity on cultures of nervous tissue have been published several methods were tested in this study combined with inhibitors with known properties

The chemical mediator for transmission of impulses from the visual cells to the nerve cells in the inner nuclear layer is unknown in vivo (for discussion see Esila 1963 Rohen 1964) Therefore also the visual cells in the explants were examined for possible AChE activity

The nonspecific esterases and other hydrolytic enzymes in the mature retina in vivo have a characteristic distribution with especially high activi ties in the visual cells (for review see Eichner 1958, Esila 1963 Rohen 1964) Therefore the retinal cultures and particularly the photoreceptor cells in the retinal cultures were investigated histochemically for such enzymes as they might indicate a cellular development

Abbreviations used AChE = Acetyl holine esterase ChE = Choline esterase ns E = Nonspecific esterases F = Esterase

MATERIAL AND METHODS

Experimental material Most of the experiment were carried out on retinal tissue from one to two day old Sprague-Dawley rats and albino rabbits In addition cultures were also prepared from embryonic rats newborn and young rats of up to four weeks age, and from young cats and mice In all about 3000 selected growing cultures were used in this study

Culture technique The original tissue culture method and its later modifications have previously been described in detail (Hansson and Sourander 1964 Hansson 1965, 1966) Briefly, small pieces of retinal tissue varying in size from a few cells up to about a cubic millimeter were explanted directly onto clean cover glasses without any collagen or plasma clot One of the problems was to ensure that the connections between the different neuroectodermal cells and their processes were formed in culture and not surviving from the organization existing in the retinae at the time of explantation Therefore the tissue pieces were dispersed mechanically into clumps of up to ten cells by being drawn repeatedly into pipettes prior to the explantation Larger tissue pieces were usually used for histochemical studies The explants were grown in Leighton tubes or in Gey chambers The nutrient medium consisted of a modified Hank s solution with high glucose concentration (500—600 mg per 100 ml) and 20 % calf serum with addition of penicillin and streptomycin

General appearance of the retinal cultures

The appearance of the retinal cultures has previously been described (Hansson and Sourander 1964) Briefly, clusters of cells grow on the surface of a monolayer of large, thin polygonal cells These latter cells which form argentophilic precollagen networks, are considered to be derived chiefly from mesenchymal cells in the blood vessels in the retina

A few of the cells on the surface of the monolayer have a phase dense cytoplasm and several long branching processes The phase light homogenous nucleoplasm in the spherical nucleus contains a single nucleolus Their perikaryon contain neurofibrils and Nissl substance Their size increases and the Nissl substance aggregates and stains more intensely during the first two weeks in vitro They are interpreted as nerve cells from the ganglion cell layer and to a certain extend also from the inner nuclear

layer of the retina Small cells of uniform size, about 7–9 μ in diameter, with polar processes Nissl substance and neurofibrils in their scanty cytoplasm and with a phase light spherical or kidney shaped nucleus stain intensely with the drug M 223 when observed in a fluorescence microscope They correspond to the bipolar nerve cells in the inner nuclear layer of the intact retina

The majority of the cells are small and of uniform size The scanty cytoplasm, just forming a thin rim around the nucleus, seems to lack neurofibrils and Nissl substance A straight process of uniform thickness and a very thin branching one emerge from opposite poles The nucleus is filled with two to six large chromatin conglomerates These cells form rosettes with a central lumen, into which the thicker processes of the cells protrude The lumen is bordered by a membranous structure These cells are interpreted as visual cells

On the surface of the monolayer there are multipolar cells with a slightly granulated cell body The oval single nucleus is difficult to observe because of the small difference in the phase density between the nucleoplasm and the cytoplasm They lack Nissl substance Their processes often show webbing at the points of branching These cells are interpreted as neuroglial cells

The processes from the different neuroectodermal cells are in connection with each other and with cell bodies New connections are continuously established while others are broken with time in vitro Most of them are of the types end to side or end to end Processes from the same nerve cell or neuroglial cell are often observed to establish a complex system of connections Nerve bundles are exceptional in the retinal cultures

A varying number of phagocytes are observed among the other cells in the explants Some of the large granulated [illegible] cells on the surface of the monolayer or among the [illegible] stained with Azur blue and metachromatic [illegible] pH They also show a [illegible] fluorescence after [illegible] treatment, indicating the presence of a [illegible]

Histochemical techniques

[illegible]

inhibitors described below were usually added to the nutrient medium half an hour before the slides were transfered to the incubation solution However, in six experimental series the explants were preincubated with the inhibitors in Holmstedt s solution without substrates for half an hour as described by Holmstedt (1957) This was done in order to compare the results obtained on living and on dead cells Most of the explants were unfixed but a few were fixed in ice cold formalin calcium The incubation times were 30 minutes to four hours at 37° C Marked diffusion artefacts were evident only after prolonged incubation of about 12 hours

Gomori s modification (1952) of Koelle s thiocholine method was also used for demonstration of AChE in both formalin fixed and unfixed explants in five experiments

The α naphthyl acetate method of Nachlas and Seligman was used as described by Barka and Anderson (1963) The substrate used was α naph thyl acetate (Sigma) with Fast Blue RR salt (Sigma) as coupling agent The incubation time at 37° C was 3—5 minutes for unfixed cultures and 30 minutes for those fixed for 5 minutes in ice cold formalin

A few experiments were performed according to the naphthol AS D acetate method (Barka and Anderson 1963)

Holt s method for substituted indoxyl acetates was also used with o acetyl 5 bromoindoxyl and o acetyl 4 chloro 5 bromoindoxyl (Sigma) as substrates (Barka and Anderson 1963)

Parallel controls were run in incubation solutions without the substrates or coupler and on cultures which had been immersed in 65° C water for 5 minutes The controls were always unstained

Inhibitors Eserine salicylate (E Merck A G, Darmstadt Germany) $1 \times 10^{-}$ M was used for inhibition of ChE AChE was inhibited by BW 284 C 51 (1 5 *bis*/N allyl N,N dimethyl 4 ammoniumphenyl/pentan-3-one dibromide Wellcome Research Laboratories, Beckenham England) at 1×10^{-5} M and ns ChE by 1×10^{-5} M IsoOMPA (tetramono*iso*propyl pyrophosphortetramide Light) 4×10^{-6} M Mipafox (N,N di*iso*propyl phosphorodiamidic fluoride Light) or in a few experiments with Holm stedt s method 3×10^{-8} M Nu 683 (dimethylcarbamate of /2 hydroxy 5 phenylbenzyl/trimethylammonium bromide, Hoffman La Roche Basel, Switzerland) E 600 (diethyl 4 nitrophenyl phosphate Mintacol solubile Bayer Leverkusen Germany) was used at a concentration of 1×10^{-5} M

Demonstration of acid phosphatases The histochemical demonstration of acid phosphatases was performed both on fixed and on unfixed retinal cultures according to the methods of Gomori and of Burstone (for tech nical details see Pearse 1960 Barka and Anderson 1963)

Demonstration of leucyl naphthyl amide hydrolase The presence of this enzym was shown as described by Nachlas *et al* using L-leucyl β naphthyl amide hydrochloride or L leucyl-4 methoxy β naphthyl amide hydrochloride (Sigma) as substrates (for technical details see Barka and Anderson 1963)

Demonstration of nucleotidases and pyrophosphatase Dinucleotidases were demonstrated according to the method of Novikoff and Goldfischer (1961) using adenosine diphosphate, inosine diphosphate and uridine di phosphate (Sigma) as substrates Pyrophosphatase was demonstrated with the same method using tiamine pyrophospate (Sigma) as substrate Adenosine triphosphatase was shown according to the method of Wachstein and Meisel (Barka and Anderson 1963) using adenosine triphosphate (Sigma) as substrate

Evaluation of the histochemical reactions obtained with the methods for esterases

The enzyme activity demonstrated with acetylthiocholine iodide as substrate and with either of the inhibitors IsoOMPA Mipafox or Nu 683 in the incubation solution, was interpreted as depending upon AChE (for discussion see Holmstedt 1957 Pearse 1960, Holmstedt and Sjoqvist 1961, Barka and Anderson 1963, Koelle 1963, Dilgenbach 1965) It was entirely inhibited by BW 284 C 51 (Table 1) Activity resistent to IsoOMPA or Mipafox but sensitive to BW 284 C 51 obtained with the other used esterase substrates was supposed to reflect AChE Ns ChE activity was demonstrated with butyrylthiocholine iodide as substrate and BW 284 C 51 as inhibitor of AChE It was inhibited with IsoOMPA and Mipafox (Table 1) The ChE activity was inhibited by eserine The eserine resistent ns E activity was further studied with E 600

The unfixed retinal cultures showed the highest activity as shown with the different histochemical methods as compared to formalin fixed explants However the latter showed the best preservation of the cellular structures In no case were the signs of diffusion marked until after pro longed incubation

inhibitors described below were usually added to the nutrient medium half an hour before the slides were transfered to the incubation solution However in six experimental series the explants were preincubated with the inhibitors in Holmstedt s solution without substrates for half an hour as described by Holmstedt (1957) This was done in order to compare the results obtained on living and on dead cells Most of the explants were unfixed but a few were fixed in ice cold formalin calcium The incubation times were 30 minutes to four hours at 37° C Marked diffusion artefacts were evident only after prolonged incubation of about 12 hours

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RESULTS

Esterase methods

Thiocholine methods The method used in most cases for demonstration of AChE activity in nerve cells in the retinal cultures of different origins was that of Holmstedt (1957) slightly modified for the tissue culture conditions The copper thiocholine complex formed (Malmgren and Sylven 1955) was precipitated as small distinct granules on the surface of the nerve cell body in the cytoplasm especially in the vicinity of the cell membrane and on its processes It was often possible to observed a single process for a long distance with the precipitate forming a string of pearls on it The discontinous staining sometimes observed did not correspond to any visible breake of the processes as observed in phase contrast microscopy Processes from different nerve cells with signs of AChE activity were observed in close connection with each other Most of the multipolar nerve cells were stained Among those which failed to stain a few showed signs of degeneration The intensity of the staining varied both between different cultures and in the same explant A number of small polar nerve cells also showed precipitates indicating AChE activity especially when observed in a phase contrast microscope (Table 2)

Table 2

Distribution of esterase activity in unfixed rabbit retinal cultures with acetylthiocholine iodide as substrate (Holmstedt's method)

Inhibitor	Multipolar nerve cells		Small nerve cells	Neuroglial cells	Rod cells		Monolayer cells	Granulated mesenchymal cells
	Cell body	Processes			Cell body	Inner segm		
No inhibitor	+	+ +	( )	—	—	—	—	(+)
Mipafox	+ +	+ +	(+)	—	—	—	—	(+)
IsoOMPA	+	+ +	(+)	—	—	—	—	(+)
BW 284 C 51	—	—	—	—	—	—	—	(+)
IsoOMPA & BW 284 C 51	—	—	—	—	—	—	—	(+)
Eserine	—	—	—	—	—	—	—	(+)
E 600	—	—	—	—	—	—	—	(+)

+ + + = Strong activity
+ + = Moderate activity
+ = Light activity
— = No activity
( ) = Activity only in some of the cells

Table 1

The effects of the inhibitors on cholinesterases

Substrate	ChE hydrolyzing		Inhibitor	ChE inhibited		ChE demonstrated	
	AChE	ns ChE		AChE	ns ChE	AChE	ns ChE
AcThCh	+	+	No inhibitor	—	—	+	+
	+	+	Mipafox	—	+	+	—
	+	+	IsoOMPA	—	+	+	—
	+	+	Nu 683	—	+	+	—
	+	+	BW 284 C 51	+	—	—	+
	+	+	IsoOMPA & BW 284 C 51	+	+	—	—
	+	+	Eserine	+	+	—	—
	+	+	E 600	+	+	—	—
BuThCh	—	+	No inhibitor	—	—	—	+
	—	+	Mipafox	—	+	—	—
	—	+	IsoOMPA	—	+	—	—
	—	+	Nu 683	—	+	—	—
	—	+	BW 284 C 51	+	—	—	+
	—	+	IsoOMPA & BW 284 C 51	+	+	—	—
	—	+	Eserine	+	+	—	—
	—	+	E 600	+	+	—	—

Abbreviations AcThCh = Acetylthiocholine iodide
BuThCh = Butyrylthiocholine iodide

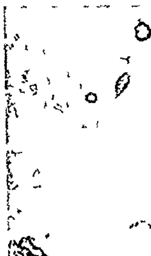


Fig 3 Acetylcholine esterase activity in a nerve cell after 35 days in culture Retinal explant from a 1 day old albino rabbit Stained according to Holmstedt with 1×10⁻⁴ M IsoOMPA as inhibitor Note the stained granular mesenchymal cell in the lower left corner of the figure Incubated for 4 hours at 37° unfixed



Fig 4 Acetylcholine esterase activity in a retinal nerve cell after 18 days in vitro from a 2 day old albino rabbit Stained according to Holmstedt using 3×10⁻⁸ M Nu 683 as inhibitor Incubated unfixed for 3 hours



Fig 5 Phase contrast microscopy picture of the nerve cell in Fig 2 showing the close r lation between the processes and the pre ipitat

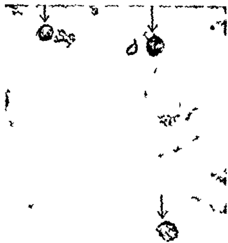


Fig 6 Nerve cells (arrows) showing acetylcholine esterase activity in a 15 day old retinal culture from a 2 day old albino rabbit Stained according to Gomori using 1×10⁻⁴ M IsoOMPA as inhibitor Incubated for 4 hours after 10 minutes fixation in cold formalin calcium

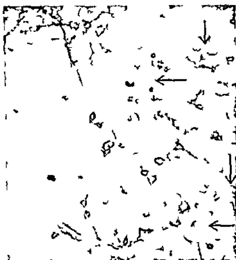


Fig 7 Retinal culture after 14 days in vitro from a 2 day old Sprague Dowley rat Stained for acetyl choline esterase activity according to Holmstedt using 1×10^{-5} M IsoOMPA as inhibitor Note the staining of the nerve cells and their processes and the unstained visual cells (arrows) Incubated unfixed for 1 hour

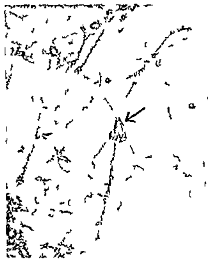


Fig 8 In the centre of the field a large multipolar nerve cell (arrow) stained for acetylcholine esterase activity according to Holmstedt using 4×10^{-6} M Mipafox as inhibitor Fixed in cold formalin calcium for 10 minutes and incubated for 4 hours The nerve cell processes show staining

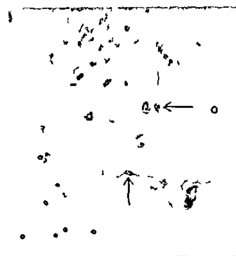


Fig 9 Nerve cells (arrows) in a retinal culture stained for acetylcholine esterase activity according to Holmstedt using 4×10^{-6} M Mipafox as inhibitor Unfixed 25 day old retinal culture from a 2 day old Sprague Dowley rat Note the stained granulated mesenchymal cells in the lower part of the picture Brightfield



Fig 10 Phase contrast microscopy picture of the same field as in Fig 9 showing the nerve cell processes

There was no significant difference in the effects of the inhibitors or in the distribution of the precipitate between those cultures preincubated with the inhibitors in the nutrient medium and those which were preincubated in Holmstedt s solution without substrate

The neuroglial cells and the visual cells remained unstained as well as the cells of the monolayer However a few of the granulated mesenchymal cells showed a slight AChE activity as previously defined (Table 1) in their cytoplasmic granules sometimes possible to observe only in phase contrast microscopy (Figs 3, 9 and 10) Such precipitates were also seen in cultures treated with BW 284 C 51 with IsoOMPA and BW 284 C 51 together and with eserine These pretreatments as well as E 600 inhibited all activity in the nerve cells (Table 2)

Ns ChE activity with butyrylthiocholine iodide as substrate and BW 284 C 51 as inhibitor of AChE was observed in the granulated mesenchymal cells A very slight activity could be seen in some of the larger nerve cells and in scattered neuroglial cells especially in old cultures after prolonged incubation

The thiocholine method of Gomori revealed AChE activity in the perikaryon but not in the processes of the nerve cells The precipitated amorphous material was of varying size and less distinct than that with the method of Holmstedt (Fig 6)

AChE activity was not observed in the nerve cells during the first week in culture The number and the size of the precipitates increased during the next week and did not change significantly thereafter This was true both for explants derived from large retinal tissue pieces and for those formed by aggregation of mechanically dispersed cells AChE activity was demonstrated in nerve cells in cultures after up to 2½ months in vitro i e the longest time examined No nuclear staining was observed

α Naphthyl acetate method The results obtained are summarized in table 3 There was no difference between the results obtained on cultured nerve cells without any inhibitor with the inhibitors IsoOMPA and BW 284 C 51 alone or together Eserine and E 600 reduced the staining intensity

The different types of nerve cells showed a moderate ns E activity However the largest multipolar nerve cells and scattered small nerve cells were heavily stained The brown precipitate was evenly distributed throughout the cytoplasm and the largest processes A weak reaction was observed in the neuroglial cells The cell body in the photoreceptor cells was unstained or revealed a very weak E activity while the proximal parts of the rod like processes corresponding to the inner segments were heavily stained

Table 3

Distribution of esterase activity in unfixed rabbit retinal cultures with α naphthyl acetate as substrate

Inhibitor	Multipolar nerve cells		Small nerve cells	Neuro glial cells	Rod cells		Mono-layer cells	Granulated mesenchymal cells
	Cell body	Processes			Cell body	Inner segm		
No inhibitor	++(+)	++	+	(+)	+	+++	+	+++
IsoOMPA	++(+)	++	+	(+)	+	+++	+	+++
BW 284 C 51	++(+)	++	+	—	+	+++	+	+++
IsoOMPA & BW 284 C 51	++(+)	+	+	—	+	+++	+	+++
Eserine	+(+)	+	(+)	—	+	++(+)	+	+++
E 600	+	—	—	—	—	+(+)	+	++

The granulated mesenchymal cells showed high E activity in their cyto plasmic granules

The relative E activity of the cell types and the dye distribution were roughly the same in unfixed and in formalin fixed cultures although the incubation time was longer in the latter case

Table 4

Distribution of esterase activity in unfixed rabbit retinal cultures with naphthol AS D acetate as substrate

Inhibitor	Multipolar nerve cell		Small nerve cells	Neuro glial cells	Rod cells		Mono layer cells	Granulated mesenchymal cells
	Cell body	Processes			Cell body	Inner segm		
No inhibitor	+(+)	(+)	(+)	—	(+)	+++	—	+++
IsoOMPA	+(+)	(+)	—	—	—	+++	—	+++
BW 284 C 51	+(+)	(+)	—	—	—	+++	—	+++
IsoOMPA & BW 284 C 51	+(+)	(+)	—	—	—	+++	—	+++
Eserine	(+)	—	—	—	—	(+)	—	+(+)
E 600	(+)	(+)	—	—	—	+(+)	—	++



Fig 11 Incubated without any inhibitor



Fig 12 Incubated with 1×10⁻⁵ M IsoOMPA

Fig 11—13 Unfixed retinal cultures after 14 days in vitro from a 2 day old albino rabbit Stained for esterase activity according to Holt using o acetyl 4 chloro 5 bromo indoxyl as substrate



Fig 13 Incubated with 1×10⁻⁵ M BW 284 C 51



Fig 14 Unfixed retinal culture from a 2 day old Sprague Dowley rat after 16 days in vitro Stained for esterase activity using α naphthyl acetate as substrate and 1×10⁻⁵ M IsoOMPA as inhibitor All the neuroectedermal cells are stained as well as a granulated mesenchymal cell (arrow)

The staining pattern described in table 3 did not change with the age of the explants in vitro although the esterase activity increased during the first three or four days in culture

Naphthol AS-D acetate method The naphthol AS D acetate method gave distinct coarse and irregular green granules They were observed in the cytoplasm often in the vicinity of the cell membrane The large nerve cell processes showed irregularly distributed, fine granules No nuclear activity was observed The distribution is described in table 4

Indoxyl acetate method Large, green crystalloid granules were obtained when o acetyl 5-bromoindoxyl was used as substrate In contrast o acetyl-4 chloro 5 bromoindoxyl as substrate gave a fine grained distinct green staining without any marked tendency for formation of large amorphous precipitate

Formalin fixation reduced markedly the rate of hydrolysis without any definite change in the localization or relative intensity of the staining (Table 5 Figs 11—13, 17, 18)

Table 5

Distribution of esterase activity in unfixed rabbit retinal cultures with o acetyl 4 chloro 5 bromoindoxyl as substrate

Inhibitor	Multipolar nerve cells		Small nerve cells	Neuro glial cells	Rod cells		Mono-layer cells	Granulated mesenchymal cells
	Cell body	Processes			Cell body	Inner segm		
No inhibitor	+++	+	++	—	(+)	+++	—	++
IsoOMPA	+(++)	+	+	—	—	+++	—	+++
BW 284 C 51	+(++)		+	—	—	+++	—	+++
IsoOMPA & BW 284 C 51	+(++)	+	+	—	—	+	—	+++
Eserine	+(+)	—	—	—	—	++	—	+++
E 600	(+)	—	—	—	—	++	—	++

It seems likely that 4 chloro 5 bromoindoxyl acetate was hydrolysed by enzymes corresponding to AChE because the staining intensity was reduced from strong to moderate by incubation with BW 284 C 51 in many but not all of the multipolar nerve cells This difference was especially pronounced in explants incubated only half of the normal time period



Fig 15 Retinal culture stained for esterases using α naphthyl acetate as substrate and eserine as inhibitor Formalin fixed for 5 minutes prior to the incubation Note the high activity in the rosettes (arrows) 12 day old culture from a 2 day old Sprague Dowley rat



Fig 16 Retinal culture stained as in Fig 15 but with E 600 as inhibitor Formalin fixed



Fig 17 Explant of retinal tissue from a 2 day old Sprague Dowley rat after 10 days in vitro stained with naphthol AS D acetate as substrate and with IsoOMPA and BW 284 C 51 in combination as inhibitor Note the strong intensity of the staining in the rosettes



Fig 18 Heavily stained granulated mesenchymal cells on the surface of a mono layer of mesenchymal cells Phase contrast microscopy of a formalin fixed 21 day old retinal culture from a 2 day old Sprague Dowley rat o Acetyl 4 chloro 5 bromo-indoxyl in combination with eserine

Acid phosphatases

The method of Burstone using naphthol AS TR phosphate AS BI phosphate and AS MX phosphate as substrates and that of Gomori using β glycerophosphate as substrate gave results in agreement with each other Details in table 6

Table 6

Histochemical activity of acid phosphatase (APhs) leucyl naphthyl amide hydrolase (LNAs) uridine dinucleotidase (UDPs) and adenosine triphosphatase (ATPs) in formalin fixed rabbit retinal cultures

Enzyme	Multipolar nerve cells		Small nerve cells	Neuroglial cells	Rod cells		Monolayer cells	Granulated *mesenchymal* cells
	Cell body	Processes			Cell body	Inner segm		
APhs	++(+)	+	+	+	+	+++	+	+++
LNAs	+(+)	—	+	(+)	—	++(+)	—	++(+)
UDPs	++	—	+	—	+	+++	+	+++
ATPs	++(+)	—	++	—	+	+++	(+)	+++

Leucyl naphthyl amide hydrolase

The distribution of leucyl naphthyl amide hydrolase was similar to that of acid phosphatases in its principle cellular distribution pattern (Table 6) The dye deposits were mostly finely granular but some diffusely distributed stain was also evident

Dinucleotidases and pyrophosphatase

The distribution of dinucleotidases is shown in table 6 There were no significant differences between the histochemical results with the substrates used i e uridine 5 diphosphate inosine 5 diphosphate and adenosine 5 diphosphate The intensity of the staining increased during the first two weeks in vitro Similar results were obtained when using thiamine pyro phosphate as substrate However the results with the latter substrate were less reproducible and often showed marked variations in the intensity of the staining The formed precipitate was less distinct with thiamine pyrophosphate than with the dinucleotides

The precipitates were fine grained after the histochemical staining for dinucleotidases Large distinct thread like deposits in the perinuclear region of the multipolar nerve cells were rarely evident and only after at least six weeks in culture in the largest nerve cells The cell membrane of the granulated mesenchymal cells were distinctly outlined after staining for dinucleotidases making it very easy to observe the complex pattern of thin expansions from these cells

Adenosine triphosphatase

The obtained results were similar to those for dinucleotidases (Table 6) No distinct perinuclear staining in the nerve cells was observed

DISCUSSION

The principal aim of this study was to investigate if AChE is present in the nerve cells including their processes in the retinal cultures Since nothing is known about the reliability of the histochemical methods for AChE when used on cultured nervous tissue, several esterase methods were used and the results compared with each other

For histochemical studies on retinal tissue from living animals it is necessary either to freeze and section or to fix embed and section the tissue to be examined However, in the cultures the cells with their membranes intact and the organelles undamaged may be immersed into the incubation solution Both unfixed and formalin fixed explants were examined histochemically in the present study for comparison of the effects of these different pretreatments Formalin fixation reduced the esterase activity in the cells in the explants but neither the distribution of the dye precipitates in the cells including the processes nor the relative intensity of the staining of the different cell types did seem to be significantly changed by these two different pretreatments

A further source of error to be avoided was the occurence of diffusion artefacts The importance of including sodium sulphate in amounts large enough to minimize the diffusion of the enzyme and of the reaction products formed in the cells during the incubation has been stressed in the literature (Gomori 1952 Holmstedt 1957 Koelle 1963) With the method of Holmstedt precipitates of copper thiocholine complex were formed in the cytoplasm especially in the vicinity of the cell membrane and along the nerve cell processes They appeared as distinct granules Careful examinations with phase contrast microscopy failed to reveal any diffusely located precipitate or precipitate not related to nerve cell structures Similar results were obtained with the thiocholine method of Gomori, although fine amorphous material was observed not only in the nerve cells but also outside the cell body just around the nerve cells There was no certain difference between unfixed and formalin fixed explants An important difference between the two thiocholine methods was that no precipitates were observed along the nerve cell processes after staining according to Gomori for AChE No nuclear activity was seen with either of these two thiocholine methods

AChE activity could be demonstrated in the nerve cells only with the

two thiocholine methods The most reproducible results were obtained with Holmstedt s modification of Koelle s method The precipitates of the copper thiocholine complex (Malmgren and Sylven 1955) were formed as distinct granules which were localized both to the nerve cell cytoplasm and to the processes but never to the nucleus Giacobini (1956) has demonstrated the same distribution of AChE activity in freshly isolated unfixed nerve cells of the central and the peripheral nervous system The results of the staining for AChE activity in the perikaryon of nerve cells including the processes are also in agreement with those for nervous tissue in vivo (Holmstedt 1957 Holmstedt and Sjoqvist 1961 Couteaux 1963 Koelle 1963 Ehinger 1966) The AChE activity in the cultured nerve cells was resistent to Mipafox IsoOMPA and Nu 683 but was completely inhibited by BW 284 C 51 eserine and E 600 (Table 2)

AChE activity was also demonstrated in the cultured retinal nerve cells after staining according to the method of Gomori However only the perikaryon and not the processes showed precipitates (Fig 6)

Indoxyl acetate seemed to be the only of the other substrates which at least partly was hydrolysed by AChE The inhibitor BW 284 C 51 caused a slight decrease in the activity in the cytoplasm and the processes of most of the nerve cells However the nerve cells showed moderate esterase activity even after treatment with IsoOMPA and BW 284 C 51 in combination which made the results unreliable (Table 3)

Thus Holmstedt s modification of Koelle s thiocholine method adapted to the tissue culture conditions was found to be suitable for demonstration of AChE activity in nerve cells in tissue cultures

In the retinal cultures AChE activity was demonstrated in the multipolar nerve cells and in some of the small nerve cells The visual cells as well as the neuroglial cells lacked AChE activity In the retina of rats rabbits mice and cats AChE activity occurs in vivo in the inner plexiform layer and in the ganglion cell layer (Esila 1963 Koelle 1963 Ehinger 1966) High activity of AChE has been demonstrated in the anterior colliculi especially in the layers of the tectum where the optic fibres terminate (for discussion see Couteaux 1963) Thus the results obtained on the retinal cultures correspond with those described to occur in vivo

Connections between different nerve cell processes of the types end to end and end to side between crossing processes and between nerve cell processes and perikaryon were often observed both in living cultures with phase contrast microscopy and in fixed and stained cultures The silver staining method of Holmes was observed to be suitable for observations and analysis of the complicated network of dendrites and axons in the

tivity in vitro was partly resistent to pretreatment with the potent inhibitor E 600 both in unfixed and in formalin fixed retinal explants The latter enzyme activity may indicate the presence of peptidases (Pearse 1960) Previously, the same localization of enzyme activity has been shown for pyrophosphatase dinucleotidases and adenosine triphosphatase (Hansson 1966) The activity of these hydrolysing enzymes increased during the first days in culture

It is tempting to speculate about the cellular structures which show these high enzyme activities The lysosomes have been stated by definition to contain acid phosphatases peptidase and esterases (Novikoff 1963) Lysosome like structures have been described in the inner segments of the visual cells in vivo (Rohen 1964, Sjostrand and Nilsson 1965) The pyrophosphatase and dinucleotidases have been localized to the Golgi complex (Novikoff and Goldfischer 1961, Torack and Barrnett 1963 Allen 1963 Shanthaveerappa and Bourne 1965) Nucleoside triphosphatase activity including adenosine triphosphatase activity has been demonstrated in the outer and inner segments of the visual cells as well as in the plexiform layers (Scarpelli and Craig 1963) Thus the high enzyme activity of the proximal part of the rod-like processes of the photoreceptor cells shown in this study may be due to the presence of lysosomes and other cytoplasmic structures with high enzyme activity The histochemical results demonstrate that the enzymatic activity of these structures increases during the first time in vitro, in the same way as in the intact retina in vivo indicating a chemical differentiation

High activities of ns E and E 600 resistent E were observed in the proximal part of the rod like processes of the photoreceptor cells both in fixed and in unfixed retinal explants These results are not in agreement with those of Esila (1963) who could demonstrate ns E in vivo only in formalin fixed nerve cells but not in unfixed In her study no E 600 resistent E activity could be shown in the inner segments of the visual cells These contradictionary results may be due to differences in the treatment of the nervous tissue Esila used cryostat microtome sections which were allowed to dry prior to the incubation In the present study the retinal cultures were placed directly into the substrate solution without any fixation freezing sectioning or drying of the tissue

Histochemical studies on the retinal cultures for pyrophosphatase and dinucleotidases revealed precipitates diffusely distributed in the perikaryon of the nerve cells The deposits were fine grained Large threadlike precipitates like those observed in vivo (Shanthaveerappa and Bourne 1965) were rarely seen in the cultured nerve cells They could occasionally be

recognized after several weeks in vitro In light and in electron micro scopic cytochemical studies the pyrophosphatase and the dinucleotidase ac tivities have been localized to the Golgi complex (Novikoff and Goldfischer 1961 Torack and Barrnett 1963 Allen 1963) Previous studies on cultured nerve cells have failed to demonstrate any lamellar network after hist ological staining resembling the Golgi apparatus (for review see Hild 1959) Studies in vivo on regenerating nerve cells in the central nervous system revealed an initial decrease in the activity of dinucleotidases and pyrophosphatase and that the lamellar structures of the Golgi complex grew thinner (Barron and Tunebay 1964, Sjostrand 1966) The cellular conditions prevailing in the retinal explants may be compared to those existing in nervous tissue during regeneration Thus the general staining pattern for enzyme activities in the cultured nerve cells seems to be the same as that described in vivo for the retinal nerve cells However the Golgi complex of the nerve cells in vitro never reached the complicated lamellar organization observed in vivo probably reflecting an incomplete development of the Golgi complex or a change in the cellular organization

Granulated cells of mesenchymal origin varying from starshaped to spherical moved around among the other cells in the retinal cultures Many of these cells showed high pinocytotic and phagocytotic activity (Hansson and Sourander 1964 Hansson 1965 1966) A varying number fulfilled the criterions for mast cells (Olsson, Hansson and Sourander 1965) The histochemical demonstrated activity for several hydrolytic enzymes were localized to the cytoplasmic granules i e acid phosphatases, ns E leucyl naphthyl amide hydrolase dinucleotidases and adenosine tri phosphatase The latter two reactions also outlined the cell membrane distinctly All the granulated mesenchymal cells showed activity of the examined hydrolytic enzymes although the number of stained granules and the staining intensity varied between different cells Previous histo chemical studies in vivo have revealed similar properties for mast cells in subcutaneous tissue The enzyme activity appeared in rat fetuses a few days before birth and increased rapidly during the next few weeks (Schauer 1964) Other studies both in vivo and in vitro have demonstrated high activity of these enzymes histochemically in macrophages (for review, see Jacoby 1965) The granulated mesenchymal cells in the retinal cultures showed an increase in the activity of the tested enzymes during the first two weeks The staining intensity did not change markedly thereafter as far as could be demonstrated in the explants if they were not subjected to treatment with chemical agents (Hansson 1966)

In conclusion it may be stated that the AChE activity was possible to

demonstrate in the retinal cultures in the nerve cells of different types including their processes after about a week in vitro At the same time there was a morphological development of the same cells The results indicate that the nerve cells, which are immature at the time of explanta tion, develop and attain several of the characteristics of mature nerve cells The same is true for the photoreceptor cells, as demonstrated by their chemical (Hansson 1966) and histochemical properties

SUMMARY

Tissue cultures of retinae from mainly young rats and rabbits were grown for histochemical demonstration of the presence of acetylcholine esterase and other hydrolytic enzymes Several different methods for esterases were used in combination with inhibitors with known properties for studies on the specificity of the histochemical results obtained on the tissue cultures

Acetylcholine esterase activity was demonstrated with the use of acetylthiocholine iodide as substrate and with either of the drugs 4×10^{-6} M Mipafox 1×10^{-5} M IsoOMPA or 3×10^{-8} M Nu 683 as inhibitors of nonspecific cholinesterases both on the cell membrane and in the cytoplasm of the multipolar nerve cells and in some small nerve cells The nerve cell processes showed high activities even at points of connections between different processes and between nerve cell processes and perikaryons Other histochemical methods with α naphthyl acetate and naphthol AS D acetate as substrates did not reveal any acetylcholine esterase activity Indoxyl acetate was probably hydrolysed to a certain degree by acetylcholine esterase

Acetylcholine esterase activity was not evident in the nerve cells until after several days in culture During the same time there was a structural development of the nerve cells The visual cells lacked acetylcholine esterase activity

The proximal parts of the rod like processes from the visual cells showed in culture a high activity for esterases acid phosphatases, peptidase leucyl naphthyl amide hydrolase dinucleotidases pyrophosphatase and adenosine triphosphatase These results are discussed and related to the conditions in vivo

Granulated cells of mesenchymal origin also showed high activities of hydrolytic enzymes

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ACTA PHYSIOLOGICA SCANDINAVICA
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AUTORADIOGRAPHIC STUDIES ON THE DISTRIBUTION OF C^{14}-LABELLED ASCORBIC ACID AND DEHYDROASCORBIC ACID

BY

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ACTA PHYSIOLOGICA SCANDINAVICA

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AUTORADIOGRAPHIC STUDIES ON THE DISTRIBUTION OF C^{14}-LABELLED ASCORBIC ACID AND DEHYDROASCORBIC ACID

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STOCKHOLM 1966

Stockholm 1966

Beckmans Tryckerier AB

:orbic acid is widely distributed in the animal organism as shown by chemi determinations The adrenal cortex the pituitary and the corpora lutea n to have the highest concentrations whereas the concentration in blood l muscles has been found to be lower than in most other tissues (*cf* Lloyd l Sinclair 1953)

For the demonstration of asorbic acid in histological sections the silver rate technique has most frequently been used (Bourne 1933 1936 Giroud l Leblond 1934 Clara 1954) The method is based upon the reduction of ver nitrate without the action of light or heat This property was considered be specific for ascorbic acid However the specificity of the method has been ongly criticized Besides ascorbic acid several other substanes in the tissues ve been claimed to reduce the silver nitrate (Clara 1954 Pearse 1961) e method has also been criticized with respect to localization since diffusion iy occur before the precipitation of the silver grains (Danielli 1946 Pearse 61)

The biochemical role for ascorbic acid in the organism is still obscure and number of miscellaneous functions have been suggested The studies have unly been performed on experimentally deficient guinea pigs which like man beings monkeys and a few other species are unable to synthesize corbic acid (Chatterjee et al 1961) In ascorbic acid deficiency it has been und that fibroblasts odontoblasts and osteoblasts loose their capacity to rm collagen There are also abnormalities in the blood vessels leading to calized haemorrhages In addition to these classical signs of scurvy several her functions in the body seem to be impaired in a deficiency state There e reports of disturbances in the metabolism of carbohydrates and of the omatic amino acids phenylalanine and tyrosine and in the resorption and position of iron (*cf* Follis 1958) Several of the symptoms have been ob rved in an advanced deficieny state when the basal metabolism of the ganism is profoundly deranged and it is difficult to distinguish the func- ons that are directly dependent on the vitamin from those changes that are condary to others or due to simple inanition

Fully developed scurvy is rarely seen nowadays but slight ascorbic acid eficiency has been found to be rather common (Czok 1965) The early symp ms of ascorbic acid deficiency are not specific and the vitamin has been ed for the therapy of several miscellaneous disorders (Ritzel 1965) Of ecial interest in dentistry is the possible relation between a subclinical scurvy d the periodontal diseases

Several analogues of ascorbic acid have been tested for antiscorbutic

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DISTRIBUTION OF ASCORBIC ACID-1 C^{14} IN ADULT MICE

The physiological requirement of ascorbic acid has been extensively investigated Yet it cannot be considered to be finally settled The amount of ascorbic acid that has been found to prevent scurvy in humans is approximately 10—20 mg a day The generally recommended daily allowance is 75 mg (Kraut 1965)

In guinea pigs the requirements seem to be considerably higher calculated on a weight basis A daily dose of 6—8 mg/kg body weight seems to prevent scurvy and 30—80 mg/kg is an abundant safe daily dose for guinea pigs (Mannering 1949)

Administered ascorbic acid is well tolerated by the organism even when it is given in large amounts In order to avoid the effects of the acidity of the vitamin large amounts are generally given in a neutralized solution or as a salt Demole (1934) gave 5 g/kg body weight orally or 1 g/kg intravenously of ascorbic acid in a neutralized solution to mice and rats without any symptoms Kieckebusch et al (1963) studied the chronic toxicity in rats of the vitamin given orally as sodium ascorbate A daily dose corresponding to 6 5 g/kg body weight was well tolerated whereas a dose of 27 3 g/kg was toxic (77 % mortality in 4 weeks) The low toxicity may in part be explained by the rapid excretion When the concentration in blood plasma in humans exceeds 1 4—2 mg/100 ml the urinary excretion of ascorbic acid rapidly increases (Friedman et al 1940)

In the present investigation 9—18 mg/kg body weight of ascorbic acid was administered intravenously to adult male mice and to pregnant female mice The distribution of the labelled ascorbic acid was studied at different intervals after injection ranging from 2 minutes to 6 days by means of whole body autoradiography (Ullberg 1954 1958) In addition the distribution in some selected organs at 5 minutes and 4 hours after injection was studied in more detail by means of microautoradiography (Hammarstrom et al 1965) Ascorbic acid is easily soluble in water and in the methods used the tissues containing the labelled vitamin have not been in contact with any aqueous solution before the autoradiographic exposure

MATERIALS AND METHODS

Labelled compound

Ascorbic acid 1 C^{14} with a specific activity of 2 4 millicuries per millimole (mci/mM) was obtained from New England Nuclear Corporation Boston,

naturally occurring isomer has the highest activity Dehydro l-ascorbic acid and some esters which metabolically can be converted to l ascorbic acid have about the same antiscorbutic activity as l-ascorbic acid[1] The activity of all other substances tested is considerably lower (Brubacher and Vuilleumier, 1965) In the animal body ascorbic acid is mainly found in the reduced state (*cf* Lloyd and Sinclair, 1953)

Administration of ascorbic acid in amounts greatly in excess of the physiological requirements causes no demonstrable effects (Kieckebusch 1963 Grab 1965 Lang 1965) whereas injection of dehydroascorbic acid causes several specific symptoms Administration of dehydroascorbic acid has been shown to cause a temporarily increased blood pressure and increased motor activity, to raise higher nervous activity for several days and to induce a temporary or permanent diabetes (Patterson 1949 1950 Patterson and Mastin 1951, Wegmann 1958 Lyhs and Tegeler 1963) This may indicate a physiological role for dehydroascorbic acid it may be the active substance in some functions generally thought to be due to ascorbic acid

The distribution of injected dehydroascorbic acid is only fragmentarily known Some quantitative studies after injection of dehydroascorbic acid have however been performed It has been shown that dehydroascorbic acid penetrates more easily into the brain and eye than does ascorbic acid (Patterson and Mastin 1951 Martin 1961 Martin and Mecca 1961, Lyhs and Tegeler 1963) After entering these tissues it seems to be rapidly reduced to ascorbic acid Dehydroascorbic acid is more lipoid soluble than ascorbic acid and it has been suggested to be the form for transport of the vitamin across cellular barriers (Patterson and Mastin 1951 Martin 1961 Martin and Mecca 1961) However this cannot be regarded as finally settled

The present autoradiographic investigation was undertaken in order to get more detailed information of the distribution of ascorbic acid and dehydroascorbic acid A better knowledge of these patterns might facilitate the understanding of the physiological functions of vitamin C

The tissue distribution in adult mice and suckling rats of C^{14} labelled ascorbic acid and dehydroascorbic acid has been studied by whole body autoradiography according to the method described by Ullberg (1954 1958) In addition more detailed studies on the distribution in some selected tissues have been made with a recently developed microautoradiographic technique (Hammarström et al 1965) In order to be able to make more quantitative estimations of the autoradiograms impulse counting has been made on some organs Finally the chemical identity of the radioactive substances at different intervals after injection has been established by thin layer chromatography A preliminary report of this work has earlier been presented (Hammarström 1965)

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MATERIALS AND METHODS

Labelled compound

Ascorbic acid-1 C^{14} with a specific activity of 2 4 millicuries per millimole (mci/mM) was obtained from New England Nuclear C

Mass USA The radioactive compound was dissolved in redistilled wate to give a concentration of 1 8 mg/ml (25 μci/ml)

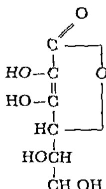


Fig 1 Structural formula of ascorbic acid The asterisk indicates the site of labelling

Animals

Adult white mice of the NMRI strain were used The male mice weighe about 20 g and the female mice which were in advanced (17—19 day pregnancy weighed about 40 g For the whole body autoradiography 5 ma and 5 pregnant female mice were used and in the microautoradiograph experiments 1 male and 1 pregnant female mouse were used

Diet

The mice were fed a complete pelleted mouse diet and water *ad lib* Th pellets were supplied by AB Ewos Sodertalje Sweden and were compose of wheypowder 5 % expanded cereals 71 % soybean oil meal 9 % fis meal defatted 8 % soybean oil 2 % yeast 2 % lime stone 1 % vitami concentrate 1 % trace elements 1 % Average analysis dry matter cruc protein 20 % crude fat 4 % ash 5 % (Ca 0 8 % P 0 6 %) fiber 3 % N fre extracts 68 % Water content 8 %

Vitamins added (per 100 grams) vitamin A 360 IU vitamin D 36 IU vitamin E 4 2 IU thiamine 150 micrograms riboflavin 300 micrograms pyri doxine 500 micrograms pantothenic acid 500 micrograms niacin 1 milligram cholin chloride 0 1 gram

Trace elements added (per cent) Fe 0 0016 Cu 0 00017 Co 0 000017 M 0 0016 I 0 00006 Zn 0 0126

According to the supplier the diet did not contain any vitamin C

Whole body autoradiography

Immediately after dissolving the ascorbic acid 1 C^{14} in the redistilled water it was injected into a tail vein of the mice Each animal was given 0 2 ml containing 0 36 mg ascorbic acid After predetermined survival periods the animals were anaesthetized with ether and then immersed into a mixture of hexane and solid CO (—75°C) The female mice were sacrificed at 2 minutes 20 minutes 4 hours 24 hours and 3 days after injection and the male mice were sacrificed at 5 minutes 60 minutes 24 hours 4 days and 6 days after injection After freezing they were mounted on a microtome stage in carboxy methyl cellulose mixed with water In a freeze room (—10°C) sagittal sections through the whole animal were then cut at different levels To ob ain whole sections an adhesive tape (No 810 Minnesota Mining and Manufacturing Co) was applied to the section surface of the frozen specimen before cutting The sections then adhered to the tape Twenty and 80 μ thick sections were taken The sections were freeze-dried at —10°C after which they were allowed to reach room temperature in an air tight box Autoradiograms were made by apposition of the sections against X-ray films (Structurix Gevaert Kodirex Kodak) After the exposure (2 weeks—2 months) the sections were separated from the film The films were developed in Gevaert G 230 and fixed in Gevaert G 305 A. Finally the sections were stained with hematoxylin and eosin or toluidine blue and mounted on glass slides in Euparate (Flatters & Garnett Ltd Manchester England) The technique has been described in detail by Ullberg (1954 1955)

Microautoradiography

One male and 1 pregnant female mouse were injected intravenously with the same dose of ascorbic acid 1-C^{14} as the mice used in the whole body autoradiography Five minutes after injection specimens were taken from the male mouse and 4 hours after injection specimens were taken from the female mouse under ether anaesthesia Specimens from the following organs were taken eye adrenal spleen ovary pancreas stomach small intestine sublingual and submaxillary salivary glands parotid gland thyroid and lung Immediately after removal of the tissue specimen they were rapidly frozen in isopentane cooled with liquid nitrogen and then freeze-dried under vacuum (10^{4} mm Hg) for 6 days After freeze drying the specimens were paraffin embedded under vacuum Five micron thick sections of the paraffin embedded tissues were taken on a cellophane tape When sectioning the tape was attached to the surface of the paraffin block The section [illegible] off adhering to the tape which thereby prevented the section from [illegible] and facilitated its further handling Three to four sections were [illegible] the same piece of tape

In the dark room nuclear emulsion plates (Ilford G 5) with an emulsion thickness of 10 microns were immersed into a solution of 10 per cent glycerin in absolute ethanol The plates were then dried in a vertical position for 5—10 minutes allowing the alcohol to evaporate The gelatin layer of the photo graphic emulsion was thus drenched with a small amount of glycerin which made it sticky enough to keep the sections attached to the emul ion surface The pieces of tape carrying sections were then applied to the plates The nuclear emulsion plates were left for exposure (1—6 weeks) under a slight pressure After exposure the G 5 plates with the adhering pieces of tape were immersed into water for 5 minutes after which the cellophane backings of the tape were removed with a pair of forceps Then the G 5 plates with the sections and the adhesive layer of the tape were passed through a series of increasing concentrations of ethanol (70 % 96 % and abs ethanol) and trans ferred to xylene During 2 hours stay in xylene the adhesive of the tape was dissolved and the G 5 plates with adhering sections were passed through a series of decreasing concentrations of ethanol (abs ethanol 96 % and 70 %) before photographic development and fixing After photographic processing and rinsing of the plates the sections were stained with hematoxylin and eosin passed through a series of increasing concentrations of ethanol transferred to xylene and mounted under coverslip with canada balsam and finally studied in the light microscope The method has previously been described (Hammarstrom et al 1965)

RESULTS

After an intravenous injection of C^{14} ascorbic acid the radioactivity rapidly left the blood and localized in the tissues As early as 2 minutes after injec tion there was a considerable concentration in the adrenal cortex choroid plexa enamel of the incisors intestinal mucosa liver and kidney (Fig 51) A rapid uptake was also observed in the pituitary in some cells in the thyroid probably the parafollicular cells and in the submaxillary salivary gland

The distribution was markedly changed with time The concentration in the liver the kidney and intestinal mucosa decreased and the concentration in the brain slowly increased After long survival periods the concentration in the brain was higher than in any other organ There was also an uptake in the ganglia but it proceeded much more rapidly than in the brain The concentration seemed to be highest in the sympathetic ganglia

In lymphatic tissues and in some endo[illegible]gans—the ovary, testis pan creatics islets and adrenal medulla—[illegible] gradual increase The con centration in those tissues was still [illegible] after injection



Fig 2 Autoradiogram of a mouse 5 minutes after an i v injection of ascorbic acid 1 C^{14} Note the uptake (light areas) in the adrenal cortex superior cervical ganglion and the enamel of the incisor In the brain hardly any radioactivity is visible except in the choroid plexa





Fig 3 ***Left*** Microautoradiogram (section + autoradiogram) of the salivary glands of a mouse 5 minutes after an i v injection of ascorbic acid 1 C^{14} Note the uptake (black grains) in the acini of the submaxillary gland The salivary ducts seem to be almost free from radioactivity like the sublingual and parotid glands Htx eosin (x 30)

Fig 4 **Right** Microautoradiogram (section + autoradiogram) of the submaxillary salivary gland of a mouse 4 hours after an i v injection of ascorbic acid 1 C^{14} Note accumulation (black grains) in the wall of ... vary duct Htx eosin (x 70)

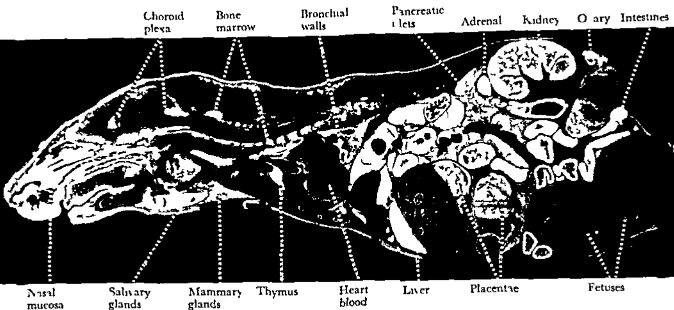
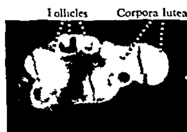


Fig 5 Autoradiogram of a pregnant mouse 4 hours after an i v injection of ascorbic acid 1 C^{14} Note the accumulation (light areas) in both the adrenal cortex and medulla *the pancreatic islets* and the ovary Note also the penetration into the brain from the periphery and from the choroid plexa The placentae have accumulated radioactivity but in the fetal tissues the concentration is low



Fig 6 Detail of Fig 5 Note the uptake in the fetal adrenals and retina Most fetal tissues have a low concentration The concentration in the fetal blood is not visible





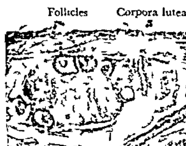


Fig 7 Left Microautoradiogram (section + autoradiogram) of the oviduct of a pregnant mouse 4 hours after an i v injection of ascorbic acid 1 C^{14} Note the uptake (black grains) in the muscular layer The mucosa seems to be devoid of radioactivity Htx eosin (x 80)

Fig 8 Right Detail of a whole body autoradiogram (upper) with the corresponding section (lower) of the ovary of a pregnant mouse 4 hours after an i v injection of ascorbic acid 1 C^{14} Note the corpora lutea follicle walls and cumulus oophorus

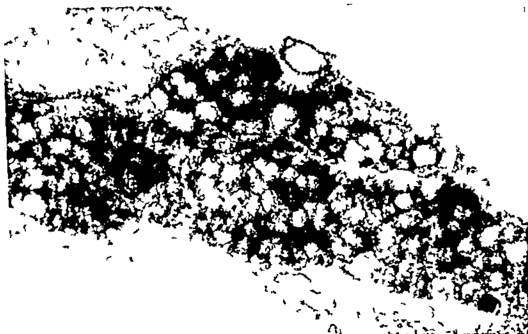


Fig 9 Microautoradiogram (section + autoradiogram) of the thyroid of a mouse 4 hours after an i v injection of ascorbic acid 1 C^{14} The highest concentration (black grains) is seen in some cells in and between the follicular walls probably the parafollicular cells There is a low concentration in the ordinary follicular cells and hardly any radioactivity in the colloids Htx eosin (x 50)

The transfer of ascorbic acid to the fetus proceeded rather slowly The fetal distribution pattern showed several similarities to that of the maternal tissues Thus the adrenal initially showed the highest concentration and after long survival periods high concentration of radioactivity was observed also in the central nervous system of the fetus

The distribution in the various tissues will be described in detail below

The circulatory system

The concentration in the *circulating blood* rapidly decreased and was very low as early as 5 minutes after injection No specific accumulation was ob served in the *walls of the blood vessels* In the *myocardium* radioactivity seemed to increase slowly but the concentration was rather low all the time Also in the *bone marrow* there was a slow increase but the maximal con centration was rather low In the *lymphatic tissues*—the white pulp of the spleen the lymph nodules and the thymus—the concentration gradually in creased and after long survival periods there was a rather high concentration compared to most other tissues except the brain and the spinal cord

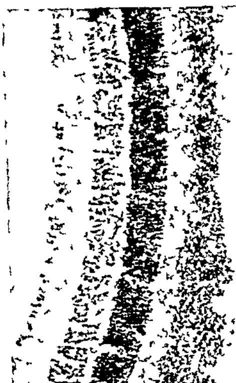




Fig 10 Left Microautoradiogram (section + autoradiogram) of a part of the eye of a mouse 5 minutes after an i v injection of ascorbic acid 1 C^{14} Note the accumulation (black grains) in the choroid No radioactivity is visible in the retina Htx eosin (x 180)

Fig 11 Right Microautoradiogram (section + autoradiogram) of a part of the eye of a mouse 4 hours after an i v injection of ascorbic acid 1 C^{14} There is an accumulation (black grains) both in the choroid and in the retina The ganglion cell layer and the inner nuclear layer seem to have the highest concentration in the retina Htx eosin (x 135)

The nervous system

The radioactivity did not penetrate directly from the blood into the *brain* In the *choroid plexa* however, a high concentration was observed as early as 2 minutes after injection After 4 hours the radioactivity could be seen in the parts adjacent to the choroid plexa and in a peripheral zone close to the arachnoid space The radioactivity continued to extend from these areas and 24 hours after injection radioactivity was observed in the whole brain The concentration was higher in the gray matter than in the white matter The highest final concentration in the brain seemed to be in the *hippocampus* and in the *cerebellar cortex* In the *spinal cord* there was a similar slow up take Also in the *eye* there seemed to be some la ency in the uptake but it proceeded more rapidly than in the brain Four hours after injection the

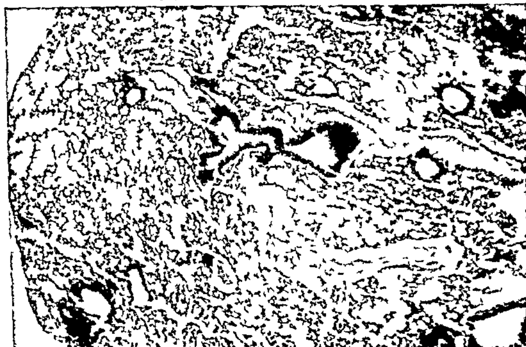


Fig 12 Microautoradiogram (section + autoradiogram) of a lung of a mouse 4 hours after an i v injection of ascorbic acid 1 C^{14} A high concentration (black grains) can be noted in the bronchial epithelium In the walls of the blood vessels no radioactivity is visible Htx eosin (x 40)

highest concentration in the eye was seen in the *choroid* and in the *retina* (Fig 10 and 11) In the retina the highest concentration was seen in the ganglion cell layer followed by the inner and outer nuclear layers Some radioactivity was found in the lens In the *pineal body* radioactivity seemed to be concentrated directly after injection

In the *peripheral nervous system* the radioactivity seemed to accumulate rapidly after injection Radioactivity was observed in the *sympathetic ganglia sensory ganglia* and also in the *myenteric plexa* in the gastro intestinal walls The concentration was highest in the sympathetic ganglia In the *peripheral nerves* the concentration was rather low

The digestive system

In the sqaumous epithelium of the *oral cavity* the *oesophagus* and the oesoph ageal part of the *gastric mucosa* an up ake was observed The secretory part of the gastric mucosa had a low concentration after short survival periods but it markedly increased with time The mucosa of the *small intestine* rapidly concentrated large amounts of the radioactivity In the mucosa of the *large*

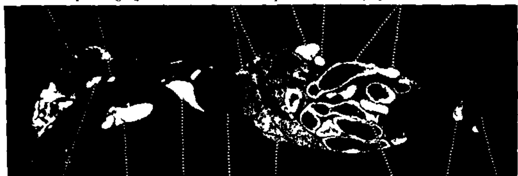


Fig 13 Autoradiogram of a male mouse 24 hours after an i v injection of ascorbic acid 1 C^{14} Note the uptake (light areas) in the brain pituitary adrenal cortex and medulla salivary glands and lymphatic tissues The testis has a low concentration

intestine the concentration was lower than in the small intestine Microautoradiography did not reveal any specific localization in the secretory mucosa of the gastro intestinal tract In the gastric mucosa the concentration was highest in the basal parts The secretion into the lumen seemed to be insignificant

The *submaxillary salivary glands* rapidly accumulated radioactivity which persisted for a long time and was still visible 6 days after injection In the *sublingual salivary glands* the radioactivity did not appear until after some

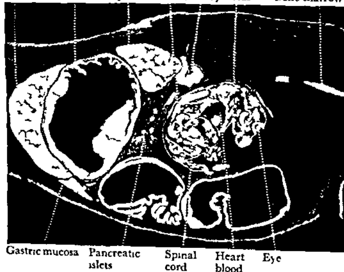


Fig 14 Detail of an autoradiogram of a pregnant mouse 24 hours after an i v injection of ascorbic acid 1 C^{14} Note the accumulation (light areas) in the ovary pancreatic islets and white pulp of the spleen In the gastric mucosa the highest concentration is found in the basal parts The adrenal the eye and the spinal cord seem to have the highest concentration of the fetal tissues in this section In the fetal blood no radioactivity is visible

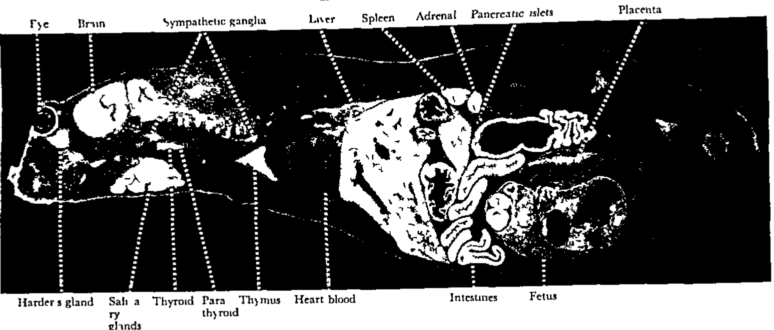


Fig 15 Autoradiogram of a pregnant mouse 3 days after an i v injection of ascorbic acid 1 C^{14} Note the accumulation (light areas) in both the maternal and fetal central nervous systems eyes and adrenals There is also an uptake in the maternal sympathetic ganglia thyroid parathyroid and pancreatic islets The sublingual salivary gland has a higher concentration than the submaxillary salivary gland No radioactivity is visible in the maternal and fetal blood

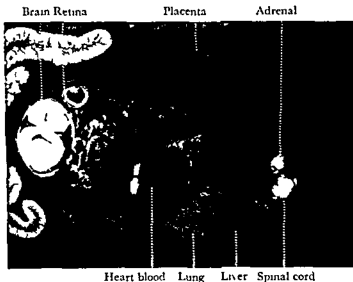


Fig 16 Detail of Fig 15 showing the distribution in the fetal tissues

latency At 5 and 20 minutes the concentration was considerably lower than in the submaxillary gland but after 1 hour and 4 hours the mucous glands had a higher concentration than the submaxillary gland After 3—4 days the differences had almost levelled out In the *parotid gland* there was a slow increase of radioactivity to a moderate concentration The microautoradiographic studies showed that 5 minutes after injection the radioactivity in the submaxillary salivary gland was localized to the acini After 4 hours an accumulation was also seen in the walls of the salivary ducts and some radioactivity was found in the lumen of the ducts (Fig 3 and 4) In the sublingual and parotid glands the distribution was more even at all the studied intervals The *liver* showed an initially high concentration which then decreased with time more rapidly than most other tissues The exocrine part of *pancreas* had a low concentration of radioactivity during the whole period of investigation After 5 minutes some radioactivity was accumulated in the pancreatic ducts

The respiratory system

The concentration in the *lungs* was initially about the same as that of the blood but the radioactivity in the lungs persisted much longer In the *bronchial tracheal* and *nasal epithelium* radioactivity was rapidly accumulated The concentration seemed to be maximal 4—24 hours after injection (Fig 12)

The endocrine system

All the endocrine organs accumulated radioactivity There were however great differences in their rate of uptake

The *pituitary* rapidly accumulated radioactivity and a high concentration persisted for a long time Four to 6 days after injection it was about the same as in the adjacent parts of the brain

In the *thyroid* there was a moderate uptake in the follicle cells In addition a marked accumulation was seen in some scattered cells probably the para follicular cells (Fig 9) These cells which were unevenly distributed in the gland rapidly accumulated radioactivity and 6 days after injection an uneven distribution of radioactivity was still observed

The *parathyroid* had a slightly higher concentration than the thyroid

In the *pancreatic islets* a rather low concentration of radioactivity was seen shortly after injection The radioactivity was unevenly distributed After longer survival periods the concentration in the islets increased and the radio activity was visible in most islet cells possibly all of them The radioactivity in the pancreatic islets was still visible 6 days after injection

In the *adrenal cortex* a rapid accumulation was observed which seemed to be about the strongest in the body Five minutes after injection the highest concentration was found in *zona glomerulosa* followed by *zona fasciculata* in a male mouse Four hours after injection an uptake was also seen in *zona reticularis* of a pregnant mouse whereas the radioactivity seemed to be low in zona glomerulosa The concentration in the adrenal cortex decreased very slowly and was still rather strong 6 days after injection The concentra tion in the *adrenal medulla* was moderate shortly after injection but it gradu ally increased After 4 hours it seemed to be about the same as in the adrenal cortex and after long survival periods it was even slightly higher

In the *ovaries* of a pregnant mouse the concentration was rather low and evenly distributed 2 minutes after injection After 20 minutes the concentra tion had considerably increased The strongest concentration was observed in *corpora lutea* and in the *walls of the follicles* Also *cumulus oophorus* and the *ova* seemed to concentrate radioactivity (Fig 8) The radioactivity in the ovary persisted for a long time and 3 days after injection it had about the same concentration as the white pulp of the spleen but less than that of the maternal and fetal central nervous systems

The *testes* had a very slow uptake The radioactivity was mainly localized in the interstitial parts

The urinary system

At short intervals after injection there was a strong concentration of radio activity in the *kidney* the *ureter* and in the *urinary bladder* After the initial excretion phase some radioactivity persisted in the renal cortex Twenty four hours after injection however the concentration in the kidney was very low

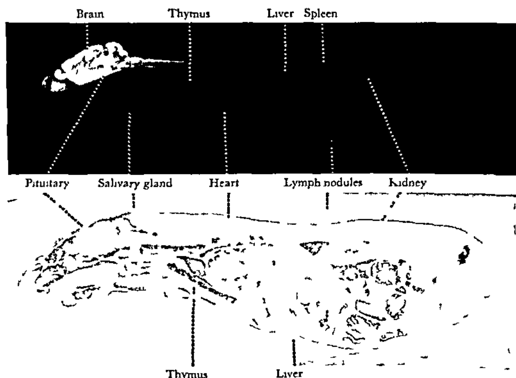


Fig 17 Autoradiogram (upper) with the corresponding section (lower) of a [illegible] after an i v injection of ascorbic acid 1 C^{14} Note the high concentration [illegible] the central nervous system Also the lymphoid organs have a higher concen[illegible] other tissues

The reproductive system

In the *testes* the radioactivity was mainly localized in th[illegible] the uptake has been described in the endocrine system T[illegible] *dymis* followed that in the testis but the concentration [illegible] *seminal vesicles* an accumulation was observed in the [illegible] distribution in the *ovary* has been described above T[illegible] moderate uptake of radioactivity in the muscular layer [illegible] observed in the mucosa (Fig 7)

Muscles

The concentration in all the muscles was rather lo[illegible] the *smooth muscles* and in the *myocardium* was hi[illegible] *muscles*

Connective tissues and hard tissues

The concentration in *fasciae* and *tendons* was rather low in the adult mice In the cells of the *pericardial pleural* and *peritoneal cavities* a high con centration was observed The cells were of different types Many of them were neutrophil leucocytes and some cells were mast cells as indicated by their metachromatic s aining in toluidine blue

In *compact bone* no radioactivity was visible except in the periosteum where a moderate concentration was observed *Cartilage* had a low concentra tion which was evenly distributed The *enamel* of the permanently growing incisors had a strong concentration throughout its thickness as early as 2 minutes after injection In the *dentin* a zone of radioactivity could be seen adjacen to the dental pulp The *pulp* of the incisors had a higher concentra tion than the circulating blood In the *periodontal membranes* a moderate uptake was noted The highest uptake seemed to be in the periosteum lining the dental alveoli

Harder s gland

This gland rapidly accumulated radioactivity to a high concentration which lasted for a long time

The epidermis

A moderate concentration was observed in the epidermis at all the intervals studied Radioactivity was especially accumulated in the hair follicles

The mammary glands

In the mammary glands of the pregnant animals a moderate concentration of radioactivity was observed at all times studied

The fetuses

The transfer of labelled ascorbic acid to the fetuses was very slow In all the pregnant mice studied the *placenta* had a moderate concen'ration Four hours after injection some radioactivity had passed to the *fetuses* and it was evenlv distributed in all the fetal tissues with some exceptions The con centration in the *fetal blood* was very low and the *fetal adrenal* and *retina* had a higher uptake than the other fetal organs (Fig 6) After 1 day and 3 days the concentration in the *fetal central nervous system* had increased (Fig 14 and 16) Three days after injection the fetal brain retina spinal cord and adrenal seemed to have a higher concentra ion than the correspond ing maternal organs In the fe al blood no radioactivity was visible There was no specific accumulation in the gastro intestinal tract the salivary glands the liver or in the kidney of the fetuses (Fig 16)

DISTRIBUTION OF DEHYDROASCORBIC ACID 1-C[14] IN ADULT MICE

It is now generally accepted that ascorbic acid normally occurs almost entirely in the reduced state in the body Banerjee et al (1952) did not find any dehydroascorbic acid in the organs investigated and Damron et al (1952) found only trace amounts of the oxidized form In muscles and blood however dehydroascorbic acid is reported to constitute 10—30 % of the total amount of vitamin C (ascorbic acid + dehydroascorbic acid) (Damron et al, 1952 Stewart et al 1953) The concentration of vitamin C in these tissues is however low and it is difficult to carry a solution of ascorbic acid through the analysis without producing small amounts of oxidized products

In scurvy the ratio of dehydroascorbic acid to ascorbic acid has been reported to increase and the ratio has been called the »scorbutic index» (Martini et al 1935) However there are conflicting data whether there is an absolute or relative increase of dehydroascorbic acid (Martini et al, 1935 Banerjee et al 1952 Damron et al 1952)

Both *in vivo* and *in vitro* experiments have shown that dehydroascorbic acid is reduced to ascorbic acid by animal tissues (Johnson and Zilva 1934 Fox and Lewy 1936 Roe and Barnum 1936 Borsook et al, 1937 Schulze et al 1938 Penney and Zilva 1943 Todhunter et al 1950 Dayton et al 1959 1966 Patterson and Mastin 1951 Martin 1961 Martin and Mecca 1961) Glutathionen seems to be involved in the reduction (Borsook et al 1937) Recently an enzyme has been shown in erythrocytes and liver which catalyses this reaction (Hughes 1964)

Injection of dehydroascorbic acid causes several specific symptoms Patterson and co workers have studied the effects of intravenously injected dehydroascorbic acid in rats Following an injection they noticed a short period of hyperactivity followed by a collapse an increased salivation and lacrimation which was sometimes followed by a porphyrin pigment a temporarily increased blood pressure and a temporary or permanent diabetes The hyperactivity could be controlled by anaesthesia The salivation and lacrimation was inhibited by atropine The blood pressure response was similar to that observed after injection of epinephrine and pretreatment with the adrenergic blocking drug Dibenamine» produced a reversal of the response Atropine sympathectomy with right splanchnictomy and low cervical cordotomy all reduced the blood pressure response Adrenalectomy did *not affect* the response The hyperactivity and the increased blood pressure as well as the salivation and lacrimation were all considered to be due to a central stimula-

in the brain after an injection of dehydroascorbic acid and the following assumed change in acidity might cause a release of acetylcholine with a resultant stimulation of the brain (Patterson 1949 1950 Patterson and Mastin 1951) The diabetogenic effect was similar to that of alloxan As is the case for alloxan the animals could be protected if they were given sulfhydryl compounds such as glutathione cysteine or dimercaptopropanol shortly before the injection of dehydroascorbic acid The induced diabetes was suggested to be the result of an irreversible combination of dehydroascorbic acid with enzymatic sulfhydryl groups (Patterson 1949 Patterson and Lazarow 1950) Recently ultrastructural changes have been reported in the pancreatic islets of the rats following diabetogenic doses of dehydroascorbic acid (Merlini and Caramia 1965) There was a degranulation of the B cells and alterations in the organization of the granular endoplasmic reticulum and the mitochondria The A- and D cells seemed not to be affected Necrosis of the B cells as produced by alloxan was not seen The authors considered that the mechanism for the diabetogenic action of dehydroascorbic acid was not the same as that of alloxan and probably not due to an inhibition of sulfhydryl groups

The vasomotor effects of dehydroascorbic acid demonstrated in rats have later been shown to occur also in cats and guinea pigs (Wegmann 1958) In addition to the central stimulation observed by Patterson and Mastin (1951) he suggested that the assumed increased acidity also might release catecholamines from the peripheral nerves and the adrenals

Some effects of dehydroascorbic acid on the central nervous system have been investigated by Lyhs and Tegeler (1963) They found that an intracardial injection of dehydroascorbic acid to guinea pigs not only produced a general neuromotor excitement but also led to a rise of the higher nervous activity lasting for several days even with animals showing markedly weak conditioned reflexes

Most of the effects of dehydroascorbic acid decreased when the substance was injected repeatedly during a short period of time This has been found to be valid also for the toxicity In rats the LD_{50} was about 320 mg/kg body weight intravenously Following a sublethal dose the rats tolerated a second dose which was 3 to 4 times as large as the initial dose After lethal doses of dehydroascorbic acid the animals died in respiratory failure similar to that observed after injection of lethal doses of epinephrine (Patterson and Mastin 1951) As little as 5 mg/kg of dehydroascorbic acid increased the blood pressure The hyperactivity was observed after a dose of 100—500 mg/kg and the diabetogenic dose was 1 100 mg/kg Due to the toxicity this dose had to be preceded by a sublethal dose

In the present investigation 9—18 mg/kg was given intravenously to a series of male and pregnant female mice The animals were killed at different times after injection ranging from 2 minutes to 4 days and whole body

autoradiograms were made In addition the distribution in some selected organs at 5 minutes and 4 hours after injection was studied by means of microautoradiography

The labelled dehydroascorbic acid was ob ained by oxidation of ascorbic acid-1 C^{14} with benzoquinone as suggested by Moll and Vieters (1936) and according to the method described by Patterson (1950)

MATERIALS AND METHODS

Labelled compounds

7 3 mg ascorbic acid-1-C^{14} with a specific activity of 2 4 mci/mM (New England Nuclear Corp) was dissolved in 4 ml redistilled water It was then shaken with an equal volume of ether containing benzoquinone (4 5 mg) The amount of benzoquinone was equivalent to the amount of ascorbic acid After shaking for 15 minutes the ether was removed and the water solution was washed 5 times with an equal volume of ether The water solution of dehydroascorbic acid 1 C^{14} was then used immediately Chromatographic control of the radiochemical purity of the solution according to the methods described later indicated that all the radioactivity represented dehydroascorbic acid 1 C^{14}

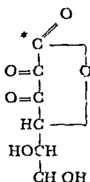


Fig 18 Structural formula of dehydroascorbic aci l The asterisk indicates the site of labelling

Animals

Three male mice weighing about 20 g and 5 female mice in advanced (17—19 days) pregnancy weighing about 40 g were used The mice were of the same strain as in the ascorbic acid 1 C^{14} experiments and they were given

Whole body autoradiography

Each mouse was given 5 μci corresponding to about 0 37 mg (9—18 mg/kg body weight) dehydroascorbic acid 1-C^{14} in 0 2 ml redistilled water intravenously in a tail vein The male mice were then sacrificed 5 and 60 minutes and 4 days after injection and the female mice were sacrificed after 2 and 20 minutes 4 and 24 hours and 3 days The animals were sacrificed under ether anaesthesia by immersion into hexane mixed with solid CO (—75°C) The sectioning and autoradiographic procedures were identical with those used for ascorbic acid 1 C^{14} The exposure time was 2 weeks—2 months

Microautoradiography

Dehydroascorbic acid 1 C^{14} of the same preparation as in the whole body experiments was used One male mouse weighing 20 g and 1 female in advanced pregnancy were each injected in a tail vein with 5 μci in 0 2 ml redistilled water corresponding to about 0 37 mg dehydroascorbic acid 1 C^{14} Five minutes after injection specimens were taken from the male mouse and 4 hours after injection specimens were taken from the female mouse The specimens were taken under ether anaesthesia and samples from the following organs were taken eye adrenal spleen ovary pancreas stomach small intestine submaxillary and sublingual salivary glands parotid gland thyroid and lung The specimens were rapidly frozen in isopentane chilled with liquid nitrogen The freeze drying and subsequent treatment were identical to that described for microautoradiography with ascorbic acid 1 C^{14} Exposure time was 1—6 weeks

RESULTS

After an intravenous injection of C^{14} dehydroascorbic acid observable amounts of the radioactivity remained in the circulating blood for several hours The concentration in the brain and the spinal cord rapidly increased and as early as 2 minutes after injection it markedly exceeded that of the blood It then remained high during the whole investigation period A rapid and persistant uptake was also observed in the ganglia the pituitary the majority of the cells in the pancreatic islets and in the thyroid In some other endocrine organs—the adrenal the ovary and the testis—radioactvity was not accumulated until after some latency Also in the lymphatic tissues there was a latency in the uptake

After 3—4 days the highest concentrations were found in the central nervous system pituitary adrenal and in lymphatic tissues At that time radioactivity was still observable also in the thyroid pancreatic islets ovary and testis

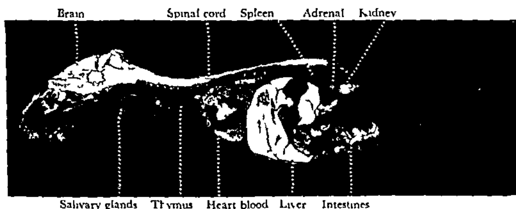


Fig 19 Autoradiogram of a mouse 5 minutes after an i v injection of dehydroascorbic acid 1 C^{14} Accumulation (light areas) is seen in the whole central nervous system The concentration is low in the adrenal cortex Radioactivity is still visible in the circulating blood

Pancreatic islets

Pancreatic duct Artery Vein

Fig 20 Microautoradiogram (section + autoradiogram) of the pancreas of a mouse 5 minutes after an i v injection of dehydroascorbic acid 1 C^{14} Note the accumulation (black grains) in the pancreatic islets in comparison with the non visible concentration in the exocrine pancreas

The transfer to the fetuses proceeded slowly and the distribution in the tal tissues was similar to that observed after injection of C^{14}-ascorbic acid The distribution in the various organs will be described in detail below

he circulatory system

fter an intravenous injection of C^{14}-dehydroascorbic acid some radioactivity :mained in the *blood* for several hours and was still observable 4 hours fter injection No specific accumulation was observed in the *walls of the lood vessels* In the *myocardium* the concentration initially was lower than iat of the blood but it seemed to increase slowly and a low concentration 'as still observable 4 days after injection In the *bone marrow* a moderate oncentration of radioactivity persisted also when the concentration in the lood was no longer observable

In the *lymphatic tissues*—the white pulp of the spleen the lymph nodules nd the thymus—the radioactivity gradually increased and persisted for a ong time

'he nervous system

mmediately after injection radioactivity was accumulated in the nervous issues—both in the *central nervous system* and in the *ganglia* A high con entration then persisted during the entire investigation The concentration vas higher in the gray matter than in the white matter but the distribution vas rather diffuse After 3—4 days the concentration in the *hippocampus* ind in the *cerebellar cortex* seemed to be higher than in other parts of the orain

The radioactivity rapidly entered the *eye* and already 2 minutes after in jection an uptake could be noted in the retina and also in the lens At 5 ninutes and 4 hours after injection the highest concentration in the retina seemed to be in the ganglion cell layer and in the inner nuclear layer

The *pineal body* had about the same concentration as the gray matter During the whole period of investigation radioactivity was accumulated in the *choroid plexa* In the peripheral nervous system the *sympathetic ganglia* had a higher concentration than the *spinal ganglia* Also in the *myenteric plexa in the gastro intestinal walls there was a rapid uptake (Fig 21 and 22)*

The digestive system

The stratified squamous epithelium of the *oral* and *oesophageal mucosa* and of the oesophageal part of the *gastric mucosa* accumulated radioactivity shortly after injection The concentration in the secretory part of the gastric mucosa was initially low but it had increased after longer periods The *intestinal mucosa* had a higher concentration than the gastric mucosa Like the con centration in the gastric mucosa it increased and was rather high 4 hours after injection The secretion into the lumen was insignificant

Fig 21 Left Microautoradiogram (section + autoradiogram) of the duodenum of a mouse 5 minutes after an i v injection of dehydroascorbic acid 1 C^{14} Note the accumulation (black grains) in the ganglia of Auerbach s plexus Htx eosin (x –7)

Fig 22 Right Detail of Fig 21 showing uptake in three ganglia of Auerbach s plexus (x 164)

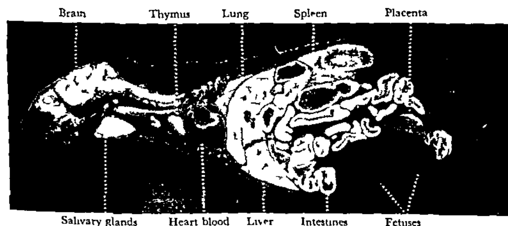


Fig 23 Autoradiogram of a pregnant mouse 4 hours after an i v injection of dehydro ascorbic acid 1 C^{14} Note the high concentration (light areas) in the brain and in the tissues

All the *salivary glands* had a low concentration shortly after injection of C^{14} dehydroascorbic acid An accumulation was however observed in the walls of the salivary ducts and some radioactivity was also found in the lumen The concentration in all the salivary glands gradually increased during the first few hours after injection The submaxillary gland seemed to reach the highest concentration

The *liver* had the highest concentration in the body shortly after injection The radioactivity then gradually left the liver and after 4 days the concentration was rather low In the exocrine part of the *pancreas* the concentration was rather low and evenly distributed after all the intervals studied

The respiratory system

The concentration in the *lungs* was initially about the same as that of the blood but the radioactivity in the lungs persisted for a longer time Four days after injection some radioactivity still could be observed in the lungs Also in the *nasal bronchial* and *tracheal epithelium* a persistant accumulation was observed

The endocrine system

The concentration in the *pituitary* was about the same as that observed in the adjacent parts of the brain during the whole investigation

In the *thyroid* a moderate concentration of evenly distributed radioactivity was observed shortly after injection In addition a strong accumulation in a few scattered cells probably the parafollicular cells was observed This unevenly distributed radioactivity persisted for a long time after injection

The concentration in the *parathyroid* was slightly higher than the average concentration in the thyroid

The *pancreatic islets* rapidly accumulated radioactivity to a rather high concentration The majority of the islet cells—possibly all of them—seemed to contain radioactivity (Fig 20)

The *adrenal cortex* had not accumulated radioactivity 2 minutes after injection of C^{14} dehydroascorbic acid Twenty minutes after injection the concentration slightly exceeded that of the blood and it then continued to increase for several hours Four hours after injection the concentration seemed to be the highest in the body The radioactivity was then mainly localized to *zona fasciculata* and *zona reticularis* The *adrenal medulla* had a moderate concentration shortly after injection which exceeded that of the cortex Like the cortex the concentration in the medulla increased Four hours after injection it was slightly lower than in the cortex Four days after injection the concentration in the cortex had decreased and the medulla had the highest concentration in the adrenal

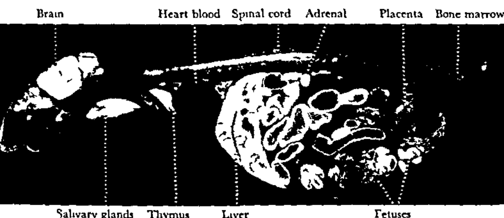


Fig 94 Autoradiogram of a pregnant mouse 24 hours after an i v injection of dehydro ascorbic acid 1 C^{14} Note the high concentration (light areas) in the maternal brain adrenal cortex and medulla salivary glands and lymphoid tissues The concentration in the fetal brain is higher than in most other fetal tissues

The *ovaries* of the pregnant mice accumulated radioactivity after some latency Four hours after injection the concentration seemed to be about the same as after injection of C^{14} ascorbic acid The localization was also similar to that of ascorbic acid

The uptake in the *testes* was slow The radioactivity was mainly localized in the interstitial parts

The urinary system

The *kidney* did not accumulate as much radioactivity as after injection of C^{14} ascorbic acid The moderate concentration in the kidney gradually decreased and after long survival periods the concentration in the kidney was low At short intervals after injection radioactivity was found in the *ureter* and *urinary bladder*

The reproductive system and the muscles

The uptake in the *reproductive system* and the *muscles* was very similar to that observed after injection of C^{14} ascorbic acid

Connective tissues and hard tissues

The uptake in most connective tissues was rather low In the *fasciae* and *tendons* a low concentration was observed In similarity wi h the distribution of C^{14} ascorbic acid some cells in the *pericardial pleural* and *peritoneal cavities* also rapidly accumulated C^{14} dehydroascorbic acid

In the *compact bone* of the adult mice no radioactivity was visible but in the *periosteum* an accumulation was observed The *dentin* accumulated radio

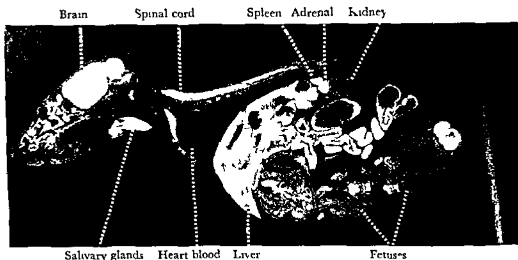


Fig 25 Autoradiogram of a pregnant mouse 3 days after an i v injection of dehydro ascorbic acid 1 C^{14} The concentration (light areas) in the fetal adrenal and brain hav now increased but still the concentration in the maternal brain and adrenal is higher

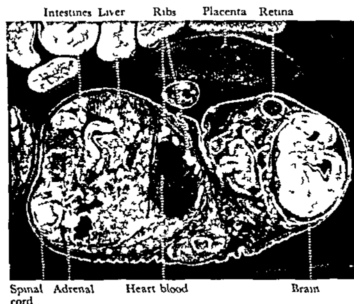


Fig 26 Detail of Fig 2 showing the distributio in the fetus Compare th distribution with Fig 1

activity in a zone adjacent to the dental pulp In the *enamel* of the growin incisor there was a latency in the uptake One hour after injection a hig concentration was observed throughout the thickness of the enamel When th radioactivity had left the circulating blood a persistant moderate concentra tion was observed in the *dental pulp* In the *periodontal membrane* the highes uptake seemed to be in the periosteum lining the alveoli

Harder's gland

Harder's gland had a high concentration of radioactivity at all intervals studied

The epidermis

In the *epidermis* a moderate concentration was observed Especially in the hair follicles radioactivity was accumulated

The mammary glands

In the *mammary glands* a moderate concentration was observed as early as 2 minutes after injection and radioactivity was still visible in these glands 3 days after injection

The fetuses

After injection of C^{14}-dehydroascorbic acid the uptake in the placentae and fetal tissues was very similar to that obeserved after injection of C^{14}-ascorbic acid and the fetal tissues slowly accumulated radioactivity The concentration in the *placenta* initially was equal to that of the maternal blood but the radio activity persisted longer in the placenta In the *fetuses* a low concentration of evenly distributed radioactivity was observed 4 hours after injection The concentration in the *fetal adrenal* and *retina* exceeded the average concentration and in the *fetal blood* no radioactivity was visible There was a slow increase in radioactivity in the *fetal central nervous tissues*

DISTRIBUTION OF ASCORBIC ACID-1-C^{14} AND DEHYDROASCORBIC ACID 1-C^{14} IN SUCKLING RATS

Vitamin C plays an important role in growth processes and young individuals are known to require more ascorbic acid calculated on weight basis than the adult (Goldsmith 1961) During infancy there is a rapid synthesis of collagen in the growing skeleton and the symptoms of scurvy seem to be more dominated by skeletal changes in young individuals than in adults (Follis 1958) In experimental scurvy young guinea pigs have frequently been used A separate investigation on the distribution of ascorbic acid and dehydroascorbic acid in growing animals therefore seemed to be motivated

C^{14} labelled ascorbic acid or dehydroascorbic acid was given intraperitoneally to 10 days old rats and intravenously to 16 days old rats Whole body autoradiograms were made and in addition a microautoradiographic investigation was made on the distribution in the head and a hind leg of the animals

MATERIALS AND METHODS

Labelled compounds

3.7 mg ascorbic acid 1 C^{14} with a specific activity of 2.4 mci/mM (New England Nuclear Corp) was dissolved in 2 ml redistilled water immediately before use

The same preparation of dehydroascorbic acid 1 C^{14} was used as in the autoradiographic investigations with adult mice

Animals

Eight 10 days old rats weighing about 20 g and six 16 days old rats of the Sprague Dawley strain weighing about 30 g were used in this investigation After injection the sucklings were left with their mothers They were given the same diet which has been described earlier

Whole body autoradiography

Four of the 10 days old rats were each injected intraperitoneally with 5 μc (0.37 mg) ascorbic acid 1 C^{14} in 0.2 ml redistilled water and the remaining four of the 10 days old rats were each injected intraperitoneally with 5 μc (0.37 mg) dehydroascorbic acid 1 C^{14} in 0.2 ml redistilled water

Fifteen minutes 2 hours 24 hours and 4 days after the injection one animal from each series was ether anaesthetized and then sacrificed by immersion into a mixture of hexane and solid CO The sectioning and subsequent whole

body autoradiography was then performed according to the methods described earlier

The 16 days old rats were also divided into two series Three rats were given 5 μci (0 37 mg) ascorbic acid 1 C^{14} and the remaining 3 were given 5 μci (0 37 mg) dehydroascorbic acid 1 C^{14} intravenously in a tail vein Five minutes 60 minutes and 24 hours after the injection one animal from each series was ether anaesthetized and then sacrificed by immersion into a mixture of hexane and solid CO Twenty micron thick sections were taken on tape No 810 (Minnesota Mining and Manufacturing Co)

The subsequent autoradiography was then performed according to the methods described earlier

Microautoradiography

From all the animals in the experiment with growing rats 5 μ thick sections of the head and a hind leg of the animals were taken in addition to the 20 μ thick whole body sections These sections were taken on tape No 688 (Minnesota Mining and Manufacturing Co) After drying in the freeze room the sections were applied to glycerin treated G 5 nuclear emulsion plates (Ilford emulsion thickness 10 μ) The glycerin treatment of the G 5 plates was made according to the method described earlier

After 2—4 weeks exposure the G 5 plates with adhering tape and sections were put in xylene 12—14 hours during which time the adhesive of the tape was dissolved and the pieces of tape fell off leaving the sections on the surface of the nuclear emulsion The G 5 plates were then passed down through an alcohol series (absolute ethanol 96 % and 70 % ethanol) before they were developed fixed and rinsed After rinsing the sections still adhering to the plates were stained with hematoxylin and eosin passed through an increasing alcohol series transferred to xylene and mounted under cover slips in canada balsam

RESULTS

The distribution patterns after intravenous injection of C^{14} ascorbic acid to young rats showed great similarities with those seen in the adult mice Thus there was a rapid accumulation of radioactivity in the adrenal cortex and a slow increase in the central nervous system Also the distribution of intravenously injected dehydroascorbic acid in the young rats was similar to that seen in the adult mice There was a rapid accumulation in the brain whereas the uptake in the adrenal cortex proceeded more slowly However the distribution patterns in the young rats were different in some respects in comparison with those of the adult mice The accumulation of radioactivity

in skeletal tissues was higher in the young rats and no specific uptake was observed in the pancreatic islets

After an intraperitoneal injection of C^{14} ascorbic acid the distribution seemed to be identical with that seen after an intravenous injection After an intraperitoneal injection of C^{14} dehydroascorbic acid however the distribution pattern was more like that of intravenously injected C^{14} ascorbic acid The rapid accumulation in the brain observed after intravenous injection of C^{14} dehydroascorbic acid did not occur Instead there was a slow increase in the central nervous system In addition to this »ascorbic acid pattern» there was a remarkably strong and long persisting concentration in the skeletal tissues (Fig 45 and 46)

The distribution of radioactivity in the hard tissues will be described more in detail below

Bone and cartilage

Both after intravenous and intraperitoneal injection of C^{14} ascorbic acid and after intravenous injection of C^{14} dehydroascorbic acid there was a rapid accumulation of radioactivity in bone and cartilage In the *cartilage* the radioactivity was evenly distributed However in the epiphyseal plates a little higher concentration could be seen in the zone of cell multiplication and in the calcifying cartilage it was markedly higher In the zone of lacunar enlargement the concentration was lower than the average

The highest concentration in *bone* was seen in the primary bone trabeculae in the metaphysis and in the periosteum and endosteum in the diaphysis (Fig 37) This distribution persisted for at least 4 days after injection There was no indication of an incorporation of radioactivity as can be seen after injection of labelled bone minerals when the primary deposition lines can be seen deep in the bone after long survival periods Also in the sutures of the bones of the skull a marked accumulation could be seen in the developing bone trabeculae (Fig 36)

The teeth

In the dental tissues there was a rapid accumulation of radioactivity after intravenous injection of C^{14} ascorbic acid whereas there was a latency in the accumulation after intravenous injection of C^{14} dehydroascorbic acid (Fig 40—43) The distribution patterns in the dental tissues however were very similar

In the developing teeth a very diversified pattern could be seen A description of the state of development of the teeth therefore seems warranted In the 10 days old rats the enamel in some cusps of the first molars had reached its full thickness as indicated by the decrease in size of the ameloblasts (Fig 34) In the other parts of the enamel of the first molars and in the

whole enamel areas of the second molars apposition of enamel matrix apparently still took place In the 16 days old rats the enamel matrix of the first molars and also in large parts of the second molars seemed to have reached the final thickness

C^{14} ascorbic acid was found to be accumulated in a superficial zone of the *enamel matrix* in those areas where deposition of matrix was still taking place In addition there wa a pronounced uptake of radioactivity throughout the thickness of the enamel in those areas where matrix formation had apparently just ceased (Fig 27 28 33 and 34) Four days after administration of C^{14} ascorbic acid to the 10 days old rats the radioactivity had left the initial accumulation areas and a sizable uptake throughout the thickness of the enamel was found more cervically in the first molars (Fig 31 and 32) At that time a marked uptake could also be seen in the cusps of the second upper molar

In the 16 days old rats no radioactivity appeared in the cusps of the first molars and in the cervical parts there was a low concentration throughout the thickness of the enamel In the second molars a substantial accumulation could be seen throughout the thickness of the main part of the enamel The third molars showed an uptake in the superficial parts of the enamel matrix (Fig 42)

In the *dentin* a zone with a moderate concentration of radioactivity could be observed immediately adjacent to the predentin The concentration in this zone decreased with time but it was localized adjacent to the predentin after all the intervals studied In addition to this localization a zone of radioactivity could also be seen in the dentin close to the dentino enamel junction in those areas where the prominent uptake in the enamel was observed As early as 5 minutes after an intravenous injection of C^{14} ascorbic acid this localization deep in the dentin was observed and the concentration was higher than in the pulpal zone of the dentin (Fig 35) In some of the sections this accumulation could be seen in the tips of the cusps where the dentin is not covered by enamel This shows that the autoradiographic blackening deep in the dentin was not due to radioactivity in the enamel (Fig 33) Some radioactivity seemed to accumulate in the proximal part of the *ameloblasts*

The *dental pulp* had a higher concentration of radioactivity than the circulating blood The radioactivity was evenly distributed with the exception of the odontoblasts which had a higher concentration than the other cells of the pulp The radioactivity seemed to be retained for a longer time in the pulps of the incisors than in those of the molars



Retina
Choroid plexus
Inner ear

Upper incisor
Molars
Lower incisor
Salivary glands

Fig 27 Upper Aut radiogram of a 10 days old rat at 2 hours after an i p injection of ascorbic acid 1 C 4 Not the accumulation (light areas) in the gastric mucosa bronchial walls and salivary gl n ls The concentration in the dental pulps is considerably higher than in the circulating bl l

Fig 28 Lower D ail of Fig 27 Note the marked accumulation in the enamel of the medial cusp of the r first molar There is al an accumulat on in the superficial layer of the dentin and amel Note also the penetration into the brain from the perip and from the choro plexus



Cerebellum
Sup cerv ganglion

Molars
Incisor
Salivary glands

Fig 29 Upper Autoradiogram of a young rat 24 hours after an i p injection of ascorbic acid 1 C[14] (The rat was 10 days old at the day of injection) Note the difference in uptake (light areas) between the sympathetic and spinal ganglia There is a high concentration in the adrenal thymus salivary glands and gastric mucosa

Fig 30 Lower Detail of Fig 29 Note the localization in the cerebellar cortex The pulps of the incisors have a higher concentration than the pulps of the molars



Brain

Upper incisor Molars Lower incis r

Fi 31 Upper Auto ogram of a young rat 4 days after an i p injection of ascorbic acid 1 C^{14} (The rat wa 10 lays old at the day of injection) The highest concentration (*light areas*) *is seen in th rat* *There i also a relatively high concentration in the testis*

Fi 39 Lower Det il f Fig 31 The accumulation in the enamel at the tip of the medial cusp of the fir u r m lar visibl in Fig 28 has now disappeared and the high concentration in the s ow lo alize l more cervically In the second upper molar a high concentration h ers d in b th cusps



Fig 33 Left side upper Microautoradiogram (section + autoradiogram) of the upper first molar of a 10 davs old rat at 2 hours after an i p injection of ascorbic acid 1 C[14] Note the high concentration (black grains) in the enamel at the tip of the medial cusp (E1) and the lower concentration in the superficial layer of the enamel matrix (E2) Note also the uptake in the dentin at the tip of the cusps (D1) An accumulation can also be seen in the dentin close to the pulp (D?) In the pulp the odontoblasts (O) have a higher concentration than the average of the pulp Htx eosin (x 40)

Fig 34 Left side lower Microautoradiogram (section + autoradiogram) of the medial cusp of the upper first molar of a 10 days old rat at 1› minutes after an i p injection of ascorbic acid 1 C[14] The exposure time has been short and the high uptake in the enamel is illustrated by the black grains at the tip of the cusp (arrow) Note the reduced height of the ameloblasts in this area indicatin~ matrix completion Htx eosin (x 121)

Fi 3 Above Microautoradiogram (section + autoradiogram) of the second upper molar of a 16 days old rat at 5 minutes after an i v injection of ascorbic acid I C[14] Note the uptake (black grains) in the enamel and the adjacent parts of the dentin The uptake in the dentin close to the predentin mav be observed although the concentration there is lower than in the other localizations of the tooth The arrows point at the two localizations in the dentin (x 9›)



Fig. 36 Microautoradiogram (section + autoradiogram) of a suture between two bones of the skull of a 10 days old rat 15 minutes after an i p injection of ascorbic acid 1 C^{14} Note the accumulation (black grains) at the ends of the trabeculae of the bone No radioactivity is visible in the connective tissue between the two bones Htx eosin (x 35)

Fig. 3 Autoradiogram of a hind leg of a 10 days old rat 2 hours after an i p injection of ascorbic acid 1 C^{14} Note the accumulation (light areas) in the epiphyseal plate in the trabeculae of the metaphysis and in the endosteum and periosteum in the diaphysis

Choroid plexus Semilunar ganglion Cerebellum Spinal ganglia

Nasal mucosa Tongue Salivary glands Thyroid Thymus Heart blood

Fi 38 Detail of an autoradiogram of a 10 days old rat ? hours after an i p injection of as orbic acid 1 C^{14} Note the accumulation (white areas) in the salivary glands spinal anglia and semilunar ganglion Note also the pattern of penetration into the brain

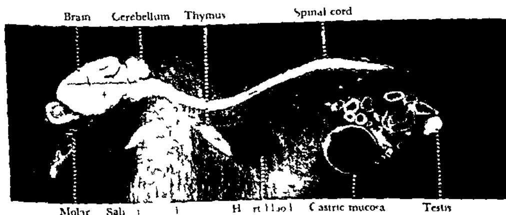


Fig 39 Autoradiog a 14 days old at 4 days after an i p injection of ascorbic acid 1 C^{14} (The rat w t injection) Note the accumulation (light areas) in the whole te also testis and thymus

Choroid plexus
Vertebrae
Teeth

Choroid plexus
Pineal body
C r b llum
Vertebrae
Teeth









Fig 40 Left side upper Detail of an autoradiogram of a 16 days old rat 5 minutes after an i v injection of ascorbic acid I C^{14} Note the accumulation (light areas) in the tooth enamel and in the choroid plexus

Fig 41 Left side lower Detail of an autoradiogram of a 1(days old rat 5 minutes after an i v injection of dehydroascorbic aci l I C^{14} Note the accumulation (light areas) in the brain choroid pl xus and pineal body In the teeth the concentration is low

Fig 42 This s i upper Detail of F g 40 (A) with the corresponding section (B) In the first molar there is a low uptake mainly in the cervical parts of the teeth In the second molars th i a considerable uptake in the whole enamel and also in the adjacent parts of the dent

Fig 43 This id lower Det l i Fig 41 (A) with the corresponding section (B) There is a low n entration in the l of the teeth



Fig 44 Autoradiogram of a 16 days old rat at 5 minutes after an i v injection of ascorbic acid 1 C[14] The picture illustrates the blood brain barrier to ascorbic acid Accumulation (light areas in the choroid plexus pineal body and pituitary Almost no radioactivity in the brain tissue Note also the difference in uptake in the salivary glands

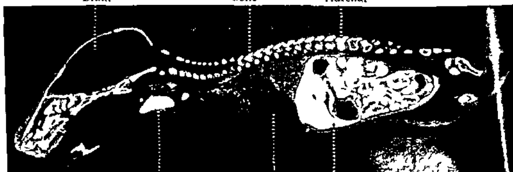


Fig 4 Autoradiogram of a 10 days old rat 2 hours after an i p inject l dehydro ascorbic acid 1 C[14] *Note the high concentration (light areas) in bone* l was no seen after the i v injection and probably indicating that some degradation proc s formed during the resorption have been accumulated there Note also the low concentration in the brain compared with the one observed after intravenous administration



Fi 4 Autoradiogram of a 14 days old rat 4 days after an i p injection of dehydro ascorbic acid 1 C[14] (The rat was 10 days old at the day of injection) Note the persisting accumulation in bone and the increased concentration in the brain

CHROMATOGRAPHIC INVESTIGATION OF SELECTED TISSUES AFTER INJECTION OF ASCORBIC ACID 1-C[14] AND DEHYDROASCORBIC ACID 1-C[14]

Ascorbic acid is metabolized in the animal organism By using ascorbic acid labelled in different positions it has been shown that a part of the molecule to some little extent may be incorporated in glycogen (Rudolff et al 1956 Chan et al 1958 Dayton et al 1959) After administration of ascorbic acid 1 C[14] however no significant amounts of radioactivity have been recovered in other components than ascorbic acid dehydroascorbic acid and their degradation products (Burns et al 1951 Curtin and King 1955 Dayton et al 1959 1966) The degradation of ascorbic acid and dehydroascorbic acid is considered to proceed to 2 3 diketo 1 gulonic acid which then may either be decarboxylated and form carbon dioxide and pentonic acids or cleaved between carbons 2 and 3 yielding oxalic acid and possibly threonic acid The catabolism of ascorbic acid has recently been reviewed by several authors (Burns 1960 Brubacher and Vuilleumier 1965 Degkwitz et al 1965)

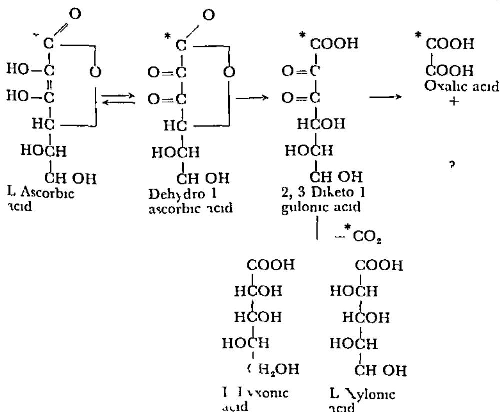


Fig 47 The most likely scheme for the catabolism of ascorbic acid The asterisk indicates the site of labelling of the preparations used in the present investigation and the labelled possible metabolite

The conversion of ascorbic acid to urinary oxalate has been shown in man guinea pig and rat The mechanism by which oxalate is formed is unknown Attempts to demonstrate signficant conversion of ascorbic acid to oxalate in rat and guinea pig tissues have not been successful (Burns 1960) The amount of ascorbic acid converted to urinary oxalate is rather small Less than 2 per cent per day of the administered ascorbic acid 1-C^{14} has been recovered as urinary C^{14}-oxalate (Burns et al 1951 1956 Lamden and Chrystowski 1954 Curtin and King 1955 Dayton et al 1959)

In both rats and guinea pigs there is a considerable conversion of ascorbic acid to carbon dioxide (Burns et al 1951 1954 Curtin and King 1955 Dayton et al 1959 1966 Salomon 1962) There are conflicting data however concerning the existence of this degradation pathway in man (Hellman and Burns 1958 Baker et al 1962 Abt et al 1963 von Schuching and Abt 1965)

Rather few qualitative tissue analysis have been made after administration of labelled ascorbic acid or dehydroascorbic acid and those studies have mainly been made 24 hours after intraperitoneal or oral administration A special study was therefore desirable to establish what the radioactivity might represent in some of the organs which showed a marked accumulation in the autoradiographic studies Thin layer chromatography was found to be a suitable method

Both ascorbic acid and dehydroascorbic acid easily decompose during the analytical procedure The decomposition is accelerated at a neutral or alkaline pH and in presence of ions of heavy metals especially those of copper and iron In absence of these metals both ascorbic acid and dehydroascorbic acid are rather stable at pH 2—3 (Prochazka 1958 Heimann and Heimann 1965) Metaphosphoric acid has been shown to have a stabilizing effect upon ascorbic acid in solution (Musulin and King 1936 Bessey 1938 Fujisawa et al 1963) In the present investigation metaphosphoric acid has been used both in the extraction solution and in the chromatographic plates The application of the radioactive samples onto the chromatographic plates as well as the chromatography have been performed under nitrogen

MATERIALS AND METHODS

Labelled compounds

Ascorbic acid 1 C^{14} with a specific activity of 6 64 mc/mM was obtained from New England Nuclear Corp 1 2 mg of the labelled ascorbic acid was dissolved in 2 0 ml redistilled water Half of it (1 0 ml) was oxidized to dehydroascorbic acid with an equivalent amout of benzoquinone (0 37 mg) in 1 0 ml ether according to the method earlier described The purity of the

radioactive substances was controlled by thin layer chromatography and sub sequent autoradiography as described below The solution of ascorbic acid 1 C^{14} showed only one radioactivity spot the R_f value of which corresponded to ascorbic acid whereas the solution of dehydroascorbic acid 1-C^{14} showed two spots One of them had an R_f value corresponding to dehydroascorbic acid containing 86 per cent of the applied radioactivity and the other spot had an R_f value corresponding to 2 3 diketogulonic acid containing 5 per cent of the applied radioactivity Nine per cent remained at the origin (Fig 48)

Animal experiments

Eight male mice weighing about 20 g were used The mice were of the same strain as those used in the autoradiographic investigations and they were given the same diet Four mice were given 0 15 mg ascorbic acid 1 C^{14} and the remaining 4 mice were given 0 15 mg dehydroascorbic acid-1 C^{14} The substances which were dissolved in redistilled water were given intravenously in a tail vein and the dose corresponded to 7 5 mg/kg body weight (6 μci per animal) At 5 minutes 4 hours 24 hours and 4 days after the injection one mouse of each series was killed by stretching the spine The adrenals liver spleen kidney salivary glands (submaxillary and sublingual salivary glands) and brain were removed and homogenized in 15 ml Potter Elvehjem homogenizers

The organs were homogenized in an extraction solution which was pre pared as follows 1 05 g citric acid was dissolved in 10 ml 1 0 N sodium hydroxide and diluted to a volume of 12 5 ml with redistilled water To this was added 25 ml 3 % metaphosphoric acid and 1 54 g trichloroacetic acid (Musulin and King 1936 Bessey 1938)

The liver spleen kidney salivary glands and brain were each homogenized in 1 0 ml and the adrenals in 0 5 ml of the extraction solution After homogenization they were centrifuged at 1 100 × g for 10 minutes Radio activity assay with a GM counter of the sediment showed that practically all of the radioactivity of the tissues had been extracted

The supernatant fluid was shaken with 0 5 ml benzene in order to avoid tailing of the chromatogram due to the extracted lipids No radioactivity appeared in this benzene phase

Chromatography

About 30 μl of each extract was spotted under nitrogen on 250 μ thick silica gel thin layer plates for chromatography The plates were prepared as described by Stahl (1962) Silica gel H and 3 % metaphosphoric acid was used for the preparation The plates were activated by heating at 120°C for 1 hour

The solvent system for thin layer chromatography found to give the b separation of possible labelled metabolites was benzene methanol aceto acetic acid (70 20 5 5) The development of the chromatogram was perforn under nitrogen The front was allowed to run approximately 16 cm Devel ment time for the chromatograms was about 50 minutes Non radioact ascorbic acid and dehydroascorbic acid were always run on each plate reference substances After the development of the chromatograms th were located by spraying the plates with 0 1 % 2 6 dichlorophenol indophe in 96 % ethanol (w/v) and 0 1 % 2 4 dinitrophenylhydrazine in 96 % etha (w/v) to which had been added 1 ml concentrated HCl

The radioactive compounds were located by autoradiography The pl were pressed against Kodirex X ray film (Kodak) with an intervening my film Exposure time was 2 months Then radioactive spots were scraped with a razor blade into liquid scintillation vials and counted in a Pack Tri Carb liquid scintillation spectrometer as a suspension in 15 ml of 0 2 5 diphenyloxazole + 0 03 % 1 4 bis 2 (4 methyl 5 phenyloxazolyl) benz + 4 % *silica gel (Aerosil) in toluene (Snyder and Stephens 1962) Cor* tion for quenching was made by using the external radiation source of spectrometer

The approximate R_f value of ascorbic acid was found to be 0 30 wh is in accordance with Stahl (1962) and the approximate R_f values of possible labelled metabolites of ascorbic acid 1-C^{14} were found to be hydroascorbic acid 0 45 2 3 diketogulonic acid 0 20 and oxalic acid 0 was not possible to obtain reproducible R_f values from plate to plate stream of nitrogen through the chromatograph chamber seemed to decre the R_f values

For the obtaining of the R_f values of the possible metabolites the follow substances were used dehydroascorbic acid prepared both with benzoquin according to Patterson (1950) and with norit according to Roe and Kuet (1943) 2 3 diketogulonic acid prepared according to Penney and Zilva (19 and finally C^{14} oxalic acid (The Radiochemical Centre Amersham Englar Ascorbic acid 1 C^{14} and dehydroascorbic acid 1 C^{14} were also used In th experiments 5—10 % of the spotted radioactivity remained at the origin

RESULTS

Only two spots except the one at the origin was obtained in all the chror tograms of extracts from the adrenal liver spleen kidney salivary gla and brain (Fig 48 and 49) These spots had the same R_f values as ascor acid and dehydroascorbic acid The main part of the radioactivity had same R_f value as ascorbic acid regardless of which one of the two vitan

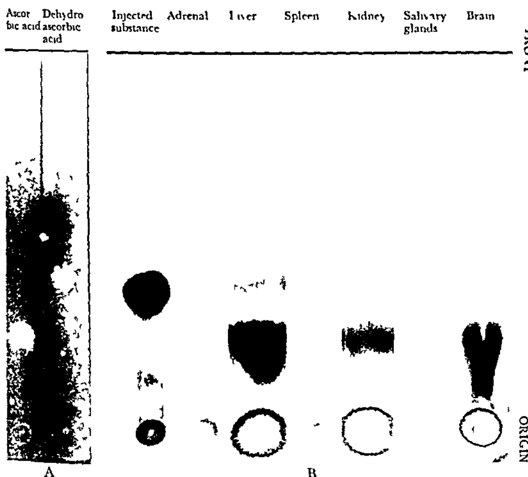


Fig 48 Radiochromatogram of tissue extracts of a mouse 5 minutes after an i v injection of dehydroascorbic acid 1 C^{14} Photograph (A) of the non radioactive reference substances after spraying with 2 6 dichlorophenolindophenol and 2 4 dinitrophenylhydrazine Autoradiogram (B) of the same plate Almost all of the injected dehydroascorbic acid 1 C^{14} has been reduced to ascorbic acid Solvent system benzene methanol aceton acetic acid (70 20 5 5)

forms that had been injected The ratio between the two spots seemed to be the same in all the tissues investigated and at all the various intervals after injection

The amount of radioactivity in most of the spots with the same R_f value as dehydroascorbic acid generally were too low to be counted with any accuracy An exception was the spots obtained from liver and kidney of the mice killed 5 minutes and 4 hours after inj ion of either one of the two vitamin forms In these samples the spots the same R_f value as dehydro ascorbic acid contained approximately per cent of the spotted radioactivity Approximately 90 per cent had the ne R_f value as ascorbic acid

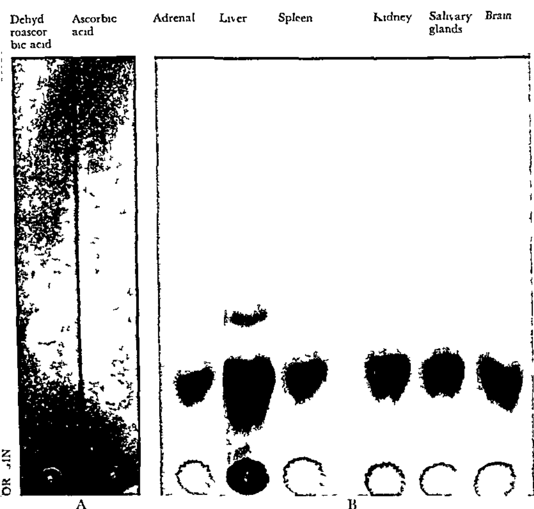


Fig 49 Radiochromatogram of tissue extracts of a mouse 4 hours after an i v injection *of ascorbic acid 1 C*[14] *Photograph (A) of the reference substances Autoradiogram (B) of* the same plate It is approximately the same ratio between the amounts of dehydroascorbic acid 1 C[14] and ascorbic acid 1 C[14] as was seen in Fig 48 Solvent system benzene methanol aceton acetic acid (70 20 5 5)

COMPARISON OF THE DISTRIBUTION PATTERNS OF ASCORBIC ACID 1 C^{14} AND DEHYDROASCORBIC ACID 1 C^{14} AS DETERMINED BY IMPULSE COUNTING

In order to be able to make a more quantitative evaluation of the autoradio graphic pictures and to facilitate the comparison of the distribution patterns of the reduced and oxidized forms of vitamin C it was desirable to measure the concentration of radioactivity in some organs of mice by impulse counting at different times after an intravenous injection of the labelled subs ances

The distribution of C^{14}-labelled ascorbic acid in the animal body has earlier been studied by means of impulse counting Burns et al (1951) studied the distribution in guinea pigs 24 hours after an oral or intraperitoneal in jection of ascorbic acid 1 C^{14} They found the highest concentration in the adrenal and the lowest in the brain kidney and heart No marked differences were found in the dis ribution of the radioactivity in the organs and tissues of normal and scorbutic guinea pigs

In the present study the adrenals brain kidney liver salivary glands and spleen were selected for impulse counting The mice were injected intra venously and different intervals from 5 minutes to 4 days after injection the animals were killed and the organs immediately removed and homoge nized For the extraction the same solution was used as in the qualitative studies Aliquots of the extracts were counted in a Packard Tri Carb liquid scintillation spectrometer

MATERIAL AND METHODS

Labelled compounds

3 7 mg ascorbic acid 1 C^{14} with a specific activi y of 2 4 mci/mM was dis solved in 9 2 ml redistilled water 5 5 ml of this solution was taken for the preparation of dehydroascorbic acid 1 C^{14} with benzoquinone according to methods earlier described Aliquots of the solutions of ascorbic acid 1 C^{14} and dehydroascorbic acid 1 C^{14} were taken for impulse counting Chromato graphic control of the purity according to the method earlier described indi cated that all the radioactivity of the two solutions represented ascorbic acid 1 C^{14} and dehydroascorbic acid 1 C^{14} respectively

Animal experiments

Sixteen male mice weighing 20—25 g wer injected with 0 2 ml (0 08 mg 1 μci) ascorbic acid 1 C^{14} and 24 male of the same weight were injec

ted with 0 2 ml (0 08 mg 1 μci) dehydroascorbic acid-1 C^{14} The substances were injected in a tail vein At 5 minutes 30 minutes, 60 minutes 2 hours 4 hours 24 hours 2 days and 4 days after injection 2 of the animals injected with ascorbic acid 1-C^{14} and 3 of the animals injected with dehydroascorbic acid 1 C^{14} were killed by stretching the spine The adrenals liver spleen kidney salivary glands (sublingual and submaxillary glands) and brain were removed weighed and homogenized in 3 ml of an extraction solution of the same composition as that used in the chromatographic investigations The corresponding organs from 2 and 3 animals respectively which were killed at the same interval after injection were pooled

Extraction and radioactivity measurements

The organs were homogenized in Potter-Elvehjem homogenizers and then centrifuged at 1 100 × g for 10 minutes Radioactivity assay was then performed by liquid scintillation of 0 1 ml aliquots of the supernatants Each 0 1 ml aliquot was added to a tricarb vial containing 3 ml ethanol and 7 ml toluene PPO (5 g diphenyloxazole in 1 l toluene) The external standard of the spectrometer showed that no correction for quenching was needed The values obtained by the liquid scintillation assays were calculated to give the concentration of radioactivity per gram of the tissue as percentages of the injected dose per gram body weight

The sediments obtained at the centrifugation of the organs were assayed in a GM counter

RESULTS AND COMMENTS

The radioactivity assays on the washed sediments showed that practically all of the radioactivity was extracted The results obtained by the liquid scintillation assays on the extracted radioactivity are condensed in the diagrams in Fig 50

The results of the impulse counting seem to be in accordance with the autoradiographic results and the differences between the distribution patterns observed in the autoradiographic investigation have been further supported

The uptake in the brain at 5 minutes after injection of C^{14} dehydroascorbic acid was 10 times that noted after injection of C^{14} ascorbic acid This ratio slowly decreased and as long as 4 days after injection there was still a difference The »C^{14} dehydroascorbic acid curve» also showed a remarkable peak at 24 hours after injection This was also noted in a similar preliminary investigation The simultaneous maximum in the »C^{14} ascorbic acid curve» indicates that it may be due to an uptake of labelled ascorbic acid emanating from the injected dehydroascorbic acid which has been reduced somewhere else in the body

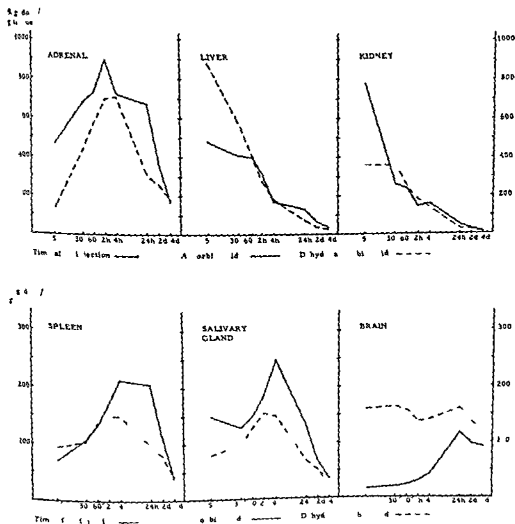


Fig. 30 Radioactivity in adrenal liver kidney spleen salivary glands (sublingual and submaxillary glands) and brain of mice at different intervals after i v injection of ascorbic acid 1 C^{14} or dehydroascorbic acid 1 C^{14} The ordinates give the contents per gram tissue as percentages of the dose given per gram animal The same dose of ascorbic acid 1 C^{14} as of dehydroascorbic acid 1 C^{14} was given

The concentration in the adrenal was found to be more than 3 times higher at 5 minutes after injection of C^{14} ascorbic acid than after C^{14} dehydro ascorbic acid The autoradiographic investigation indicated a still greater difference at 2 minutes after injection The uptake in the adrenal medulla seems to be the same regardless of which one of the two compounds that had been injected and the difference in concentration in the whole adrenal apparently reflects the different concentrations in the adrenal cortex





Fi 51 Upper Autoradiogram of a pregnant mouse 2 minutes after an i v injection of asco bic acid 1 C^{14}

Fig 52 Lower Autoradiogram of a p egnant mouse 5 minutes after an i v injection of dehydroascorbic acid 1 C^{14}

Note the differences a high concentration (light areas) in the adrenal cortex and no visible uptake in the brain except in the choroid plexa after injection of ascorbic acid 1 C^{14} a very low concentration in the adrenal cortex and a high concentration in the brain after injection of dehydroascorbic acid 1 C^{14} The concentration is low in the spleen of both animals and no transfer across the placenta seems to have occurred

In addition to the central nervous system there was also a more pronounced accumulation in the liver of C^{14} dehydroascorbic acid than of C^{14} ascorbic acid In the present study the uptake at 5 minutes after injection of C^{14} dehydroascorbic acid was twice that noted after C^{14}-ascorbic acid In the kidney the uptake was quite the contrary The concentra ion at 5 minutes after injection of C^{14} ascorbic acid was twice that of C^{14} dehydroascorbic acid The

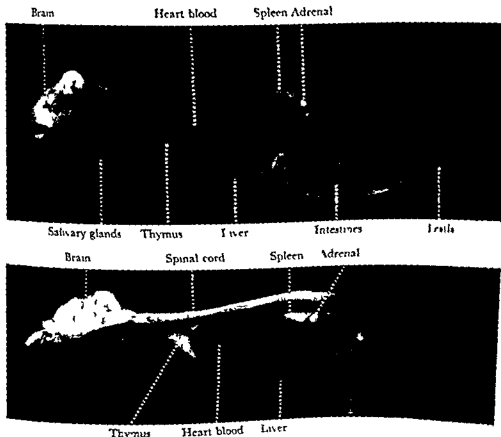


Fig 53 Upper Autoradiogram of a mouse 4 days after an i v inj [illegible] acid 1 C^{14}

Fig 54 Lower Autoradiogram of a mouse 4 days after an i v inj [illegible] ascorbic acid 1 C^{14}

The distribution patterns are now very similar Note the persisting [illegible] in the brain adrenal and lymphatic tissues

radioactivity left both the liver and the kidney more [illegible] than most other tissues The differences in uptake as well as the [illegible] are in agreement with the autoradiographic investigations

The rather complicated curve of C^{14} ascorbic acid in the salivary glands may be put in relation to the autoradiographic findings The submaxillary gland rapidly showed a marked concentration of [illegible] The radioactivity in this gland then seemed to decrease [illegible] while there was a slow uptake in the sublingual gland which [illegible] after injection This seems to be in good agreement [illegible] diagram

The uptake in the spleen seemed to proceed with [illegible] of which one of the two forms that had been injected

To summarize some of the differences in rate of uptake of ascorbic acid 1-C^{14} and dehydroascorbic acid 1-C^{14} by the various tissues noted in the autoradiographic investigation and at the impulse counting Ascorbic acid 1 C^{14} was more rapidly taken up in the adrenal cortex kidneys intestinal mucosa and in the developing enamel Dehydroascorbic acid 1 C^{14} was more rapidly accumulated in the central nervous system the liver and in the pancreatic islets of adult mice It should however be noted that neither C^{14} dehydroascorbic acid nor C^{14} ascorbic acid were specifically accumulated in the pancreatic islets of young animals

The great differences seem to occur only after intravenous injection as indicated by the distribution pattern of intraperitoneally injected dehydroascorbic acid 1-C^{14}

DISCUSSION

The chromatographic investigation indicated that after injection of ascorbic acid 1 C^{14} the radioactivity remaining in the body mainly represented non metabolized ascorbic acid and labelled degradation products were evidently rapidly excreted which is in agreement with earlier data (Burns et al 1951 Curtin and King 1955) Also after an intravenous injection of dehydro ascorbic acid 1 C^{14} the radioactivity in the tissues almost exclusively seemed to represent ascorbic acid This is in accordance with the results of Dayton et al (1966) who analysed the liver spleen, adrenals testes and kidneys of guinea pigs and rats 24 hours after oral or intraperitoneal administration of dehydroascorbic acid 1 C^{14} and found at least 95 per cent of the radio activity in these tissues to represent ascorbic acid

The chromatographic results are also in agreement with the findings by Patterson and Mastin (1951) who found a rapid increase of the reduced ascorbic acid concentration in the brain and eye after intravenous injection of dehydroascorbic acid

The reduction seemed to occur very rapidly in the brain liver kidney and submaxillary salivary glands It is however uncertain whether dehydro ascorbic acid was also reduced by the adrenal cortex and spleen since the accumulation of radioactivity in these tissues was slow and the accumulated ascorbic acid 1 C^{14} might have been reduced in some other tissues *In vitro* studies have shown that dehydroascorbic acid is reduced by the kidney liver and intestinal mucosa of rats and by the liver muscle small intestine and erythrocytes of guinea pigs (Borsook et al 1937 Schultze et al 1938)

The ratio between the concentrations of dehydroascorbic acid and ascorbic acid which was found after an intravenous injection of any one of the two substances agrees well with the concentration ratios in the tissues of guinea pigs reported by Damron et al (1952) Still it is uncertain however whether these small amounts of dehydroascorbic acid actually are present in the tissues or they are formed during the analytical procedure

In the chromatographic investigations of the tissue extracts up to 10 per cent of the spotted radioactivity remained at the origin and might represent oxalic acid This seems however unlikely since this amount remained at the origin also when the injection solutions of ascorbic acid 1 C^{14} and dehydro ascorbic acid 1 C^{14} were chromatographed and in the animals there was no indication of a distribution pattern similar to that seen after injection of C^{14} oxalic acid (Fig 55) (Hammarstrom unpublished)

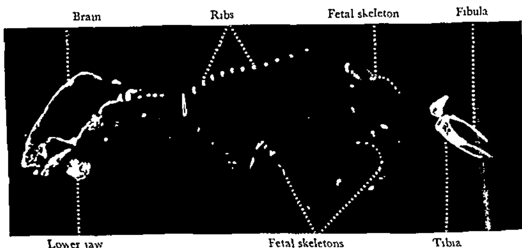


Fig 55 Autoradiogram of a pregnant mouse 4 hours after an i v injection of C^{14} oxalic acid There is a high concentration (light areas) both in the maternal and fetal skeletons In the other tissues the radioactivity is hardly visible (Hammarstrom unpublished)

After an intraperitoneal injection of ascorbic acid-1 C^{14} the autoradiographic distribution pattern was very similar to that seen after an intravenous injection When dehydroascorbic acid 1 C^{14} was given intraperitoneally however the distribution pattern was very different from that seen after the intravenous injections Part of the administered dehydroascorbic acid seems to have been reduced to ascorbic acid during the resorption as indicated by the slow increase of radioactivity in the brain This is consistent with the fact that all the pharmacological effects of dehydroascorbic acid have been noted after injection of dehydroascorbic acid directly into the blood (Patterson 1949 1950 Patterson and Mastin 1951 Wegmann 1958 Lyns and Tegeler 1963) A degradation of some of the labelled dehydroascorbic acid seems also to have occurred possibly to C^{14} oxalic acid as indicated by the high accumulation in bone Considerably more urinary C^{14} oxalate has been found after an intraperitoneal injection of dehydroascorbic acid 1 C^{14} than of ascorbic acid 1 C^{14} (Curtin and King 1955 Dayton et al 1959) This may indicate that dehydroascorbic acid is not reduced in the peritoneal fluid It has earlier been shown that dehydroascorbic acid is not reduced in the blood plasma nor in the cerebrospinal fluid (Plaut and Bulow 1935 Borsook et al 1937 Schultze et al 1938)

Dehydroascorbic acid is considered to be more lipoid soluble than ascorbic acid and has been suggested to be the form for transport across cellular membranes (Patterson and Mastin 1951) The oxidation of ascorbic acid to dehydroascorbic acid was suggested to occur in the kidney and possibly in the intestines (Martin 1961 Martin and Mecca 1961)

The distribution patterns of the two vitamin forms give little if any support

to these theories As a matter of fact the radioactivity seemed to leave the circulating blood more slowly after an injection of dehydroascorbic acid 1 C^{14} than after ascorbic acid 1 C^{14}

The labelled dehydroascorbic acid apparently entered the brain directly from the blood vessels whereas there seemed to be a complete blood brain barrier to the reduced form If ascorbic acid had been oxidized somewhere else in the body for the transport into the brain there should have been some penetration of the radioactivity directly from the blood vessels after injection of C^{14} ascorbic acid This does not exclude the possibility that ascorbic acid is oxidized to dehydroascorbic acid in the choroid plexa or in the cerebro spinal fluid for the penetration into the brain According to Plaut and Bülow (1935) however ascorbic acid occurs entirely in the reduced form in the cerebrospinal fluid in man and rabbit

The adrenal cortex was not able to accumulate dehydroascorbic acid where as labelled ascorbic acid was rapidly taken up there This difference in accumulation makes it less probable that dehydroascorbic acid is the physiological form for transportation of vitamin C into the cells of the adrenal cortex

In several other tissues the rate of uptake seemed to be independent of which one of the two forms that was administered In these tissues—e g lymphoid tissues ganglia testes —there was no indication whether it was dehydroascorbic acid or ascorbic acid that actually entered the cells The placental transfer of radioactivity was slow independently of which one of the two vitamin forms that had been injected and the distribution pattern in the fetal tissues was similar to that seen in the adult animals after injection of ascorbic acid 1 C^{14} This may indicate that ascorbic acid is the form for the placental transfer which also argues against the theory of Martin and Mecca (1961)

The results of the autoradiographic investigation seem to be in accordance with most earlier histochemical studies on the localization of vitamin C, reviewed by Clara (1954) The histochemical localization of ascorbic acid in the central and peripheral nervous system adrenal cortex and medulla ovary, testis pancreatic islets were also observed in the present autoradiographic investigation Also in cartilage bone and dentin the positive histochemical reactions seem to have been specific for vitamin C (Bourne 1956 Naujoks 1956 Harndt 1966) In some investigations by Clara (1954) and others a strong positive histochemical reaction has been obtained in the capsule of the adrenal and in the tissues surrounding the lymph nodules of the gastro intestinal tract There was no indication of such a distribution in the present investigation and it indicates that some diffusion may occur in the silver nitrate technique A comparison of histochemical and autoradiographic results will be made in the discussion of the distribution in the various organ systems

Both ascorbic acid and dehydroascorbic acid have been shown to take part in the synthesis of some biogenic amines For the hydroxylation of tryptophan to 5 hydroxytrophan—the precursor of serotonin—dehydroascorbic acid is probably required The possible mechanism of this hydroxylation is that a hydride ion in the 5 position of the tryptophan ring is replaced by a hydroxyl ion from the water The resulting two protons and two electrons then probably reduce dehydroascorbic acid to ascorbic acid (Cooper, 1961 Cooper and Melcer 1961) Hydroxylation of tryptophan to 5 hydroxytrypto phan has been demonstrated *in vitro* in the intestinal mucosa liver and to a small extent in the kidney (Cooper 1961 Cooper and Melcer 1961 Freed land et al 1961) It has also been demonstrated in the mammalian brain both *in vivo* and *in vitro* (Gal et al 1963 Grahame Smith 1964 Consolo et al 1965 Green and Sawyer 1966)

Ascorbic acid has been shown to be a co factor at the hydroxylation of dopamine to norepinephrine This reaction requires molecular oxygen and ascorbic acid which functions as an electron donor is oxidized to dehydro ascorbic acid (Levin et al 1960 Kaufman and Friedman 1965 Kaufman 1966)

It is interesting to note that several tissues where dehydroascorbic acid and ascorbic acid were accumulated are probable sites of synthesis of these amines For example the localization in the central nervous system and in the ganglia may be put in relation to these functions In the endocrine organs labelled 3 4 dihydroxyphenylalanine has been found to accumulate in the parathyroid the parafollicular cells of the thyroid the adrenal medulla and in some few cells in the pancreatic islets possibly the A cells (Hammarstrom Johansson Ullberg in preparation) which are all tissues where ascorbic acid 1 C^{14} seemed to be taken up Labelled 5 hydroxytryptophan is taken up in the adrenal medulla the parafollicular cells of the thyroid and in the B cells of the pancreatic islets (Ritzen et al 1965) where also dehydro ascorbic acid was rapidly accumulated

The main function of vitamin C in the metabolism of collagen seems to be a co factor in the hydroxylation of proline to hydroxyproline and possibly also of lysine to hydroxylysine (Gould and Woessner 1957 Robertson 1964 Udenfriend 1966) Like the β hydroxylation of dopamine also these reactions require molecular oxygen and ascorbic acid is the form needed (Fujimoto and Tamiya 1962 Prockop et al 1962)

In the present investigation there was a low concentration of radioactivity in the proper connective tissue This type of collagen is little sensitive to ascorbic acid deficiency (Gould 1961) It is probably due to the slow rate of metabolism (Neuberger and Slack 1953 Robertson 1961) In the hard tissues of the young animals however a higher concentration of C^{14}-ascorbic acid was seen rapidly after injection This may be put in relation to the

synthesis of hydroxyproline and hydroxylysine, which suggests these reactions to be directly implicated in the mineralization process

It may be of interest to correlate more in detail the distribution patterns of ascorbic acid-1 C^{14} and dehydroascorbic acid 1-C^{14} and their demonstrated biochemical functions with the various symptoms of a deficiency of vitamin C

The nervous system

Rather little work has been done on the physiological relations between ascorbic acid and the central nervous system Both a decreased and an increased concentration seem to affec the normal function of the brain In experimental ascorbic acid deficiency in man Crandon et al (1940) noted an extreme fatigue as an early sign of scurvy Monkeys fed a diet deficient in vitamin C became debile (Shaw 1949) Also in guinea pigs an impaired nervous activity has been noted (Follis 1958)

If the concentration of ascorbic acid in the brain is increased by injection of dehydroascorbic acid an intense hyperactivity of short duration has been noted (Patterson and Mastin 1951) and also a rise of the higher nervous activity lasting for several days (Lyhs and Tegeler 1963) The relation between the deficiency symptoms the effects of an increased concentration of ascorbic acid and the synthesis of serotonin and catecholamines in the brain is not known

Most of the monoamine containing cells in the brain seem to be localized in the brain stem (Vogt 1954 Bertler and Rosengren 1959 Carlsson et al 1962 Dahlström and Fuxe 1965)

The whole rat brain has been found to contain appreciable amounts of dopamine β hydroxylase *in vitro* The highest enzymic activity seems to be in hypothalamus and caudate nucleus little in cerebellum and no detectable activity in cortex (Udenfriend and Creveling 1959) Masuoka et al (1963) found tyrosine to be converted to norepinephrine by cat brain slices from hypothalamus visual cortex and reticular area but not from caudate nucleus

The bulk of the tryptophan hydroxylase activity in the brain seems to be localized in the brain stem as indicated by *in vitro* studies on rat brain homogenates No activity could be demonstrated in cerebellum (Green and Sawyer 1966)

When the C^{14} labelled precursors 3 4 dihydroxyphenylalanine or 5 hydroxy tryptophan were injected there was a rather even distribution of radio activity in almost all the brain (Rosell et al 1963 Ritzen et al 1965) Both the subs ances showed a higher concentration in the choroid plexa and in the pineal body than elsewhere in the brain Ritzen et al 1965 Hammar strom Johansson Ullberg in preparation)

According to the autoradiograms ascorbic acid was accumulated in almost all the brain tissue but the highest concentrations seemed to be in the hippo

campus and the cerebellar cortex Apparently there is little agreement with the areas where the main synthesis and localization of biogenic monoamines have been demonstrated and the localization of the highest concentration of ascorbic acid

Histochemical studies with the acid silver nitrate technique of Giroud and Leblond (1934) indicate that all the nerve cells of the central nervous system contain ascorbic acid (Clara 1954) The positive reaction was obtained in the cell bodies and only occasionally in the dendrites There were great variations in the concentration of ascorbic acid be ween the various cell types and also between the cells of the same type Strong positive reactions were obtained in cells in the cerebral cortex striatum thalamus cerebellar cortex and in certain cells in other parts of the brain In the vegetative nuclei there were cells with a strong positive reaction (Clara 1954) The results of these histochemical studies seem to be in agreement with the autoradiographic distribution pictures However a more detailed autoradio graphic investigation is necessary if the localization and concentration of ascorbic acid in all the indicated areas are to be discussed

The mode of uptake of ascorbic acid in the brain is of special interest since there appeared to be a complete blood brain barrier to the reduced form whereas the oxidized form easily passed directly from the blood vessels into the brain The existence and localization of a blood brain barrier has caused considerable debate (*cf* Dobbing 1961) The localization of the barrier for ascorbic acid seemed to be the same as that observed for trypan blue (King 1939 Wislocki and Leduc 1952) The pituitary the pineal body *and the choroid plexa are outside the barrier and they rapidly accumulated* the radioactivity of ascorbic acid 1 C^{14} There seems to be a similar blood brain barrier to the catecholamines and serotonin (Bertler et al 1966 Ham berger and Hamberger 1966)

From the *choroid plexa the radioactivity of C^{14}-ascorbic acid seemed to* enter the brain possibly together with simultaneously secreted cerebrospinal fluid and then proceed *per continuitatem* into the brain The concentration of ascorbic acid in the cerebrospinal fluid of rabbits is almost twice that of the blood plasma (Plaut and Bulow 1935)

There seemed to be a similar barrier to the penetration of ascorbic acid into the eye and there was some latency before the radioactivity of C^{14} ascorbic acid was visible in the retina It is interesting to note that ascorbic acid has been shown to enter the eye as a result of a secretory process and seems to be involved in the secretion of the intraocular fluid (Friedenwald et al 1943 Kinsey 1950)

Recently adrenergic structures have been demonstrated in three zones in the inner plexiform layer of the retina of mice and several other species (Malmfors 1963 Häggendahl and Malmfors 1963 1965 Ehinger 1966) This

may be correlated to the marked uptake of ascorbic acid in the ganglion cell layer and the inner nuclear layer

In the peripheral nervous system the radioactivity of labelled ascorbic acid as well as dehydroascorbic acid was taken up in significant amounts in the ganglia and the highest concentration was found in the sympathetic ganglia This is consistent with the histochemical results in humans where a substance capable of reducing acid silver nitrate was found in almost all the nerve cells of the sympathetic ganglia and in some few cells in the sensory ganglia In rats it is necessary to give ascorbic acid parenterally for the demonstration of the localization in the spinal ganglia (*cf* Clara 1954)

Catecholamines mainly norepinephrine have been demonstrated in the autonomic ganglia but so far not in the sensory ganglia After injection of C^{14}-labelled 3 4 dihydroxyphenylalanine however there was not only a marked uptake of radioactivity in the sympathetic ganglia but also a moderate accumulation in the spinal ganglia (Hammarström Johansson Ullberg in preparation)

In the ganglia of Auerbach s plexus in the gastro intestinal walls both the reduced and oxidized ascorbic acid was rapidly accumulated These ganglia have been shown by autoradiography to accumulate 3 4 dihydroxyphenylalanine H^3 and 5 hydroxytryptophan H^3 (Gershon et al 1965 Hammarstrom et al 1966 Hammarstrom Johansson Ullberg in preparation) Adrenergic structures in these ganglia have also been demonstrated by fluorescence microscopy (Norberg 1964 Hollands and Vanov 1965)

The circulatory system

Petechial hemorrhage is a prominent symptom of ascorbic acid deficiency Little is known about the biochemical mechanism behind this defect and it is generally explained by an assumed lack of an endothelial cement substance Injection of a dye to vitamin C deficient guinea pigs indicates however that there is no increased vascular permeability (Fister and Schack 1950) Recent investigations have shown that ascorbic acid is probably involved in the maintenance of the tone of the blood vessels (Lee 1961) This is consistent with the present investigation since no specific uptake of the reduced or oxidized form of ascorbic acid was demonstrable in the walls of the blood vessels whereas a considerable concentration was noted in the sympathetic ganglia The temporarily increased blood pressure after injection of dehydroascorbic acid may also be noted in this connection (Patterson and Mastin 1951 Wegmann 1958)

The blood platelets contain large amounts of serotonin and the possible role of dehydroascorbic acid in the synthesis of this amine may also be related to the scorbutic hemorrhages Serotonin has been claimed to increase the capillary resistance both in man and experimental animals and to reduce

the bleeding time The results in this field are however contradictory (*cf* Erspamer 1961)

In the present investigation the radioactivity seemed to persist longer in the blood after injection of dehydroascorbic acid 1 C^{14} than after ascorbic acid 1-C^{14} This may be put in relation to the penetration of dehydroascorbic acid into erythrocytes *in vitro* which has been noted (Lloyd and Sinclair 1953)

The concentration of ascorbic acid in leucocytes and blood platelets has been reported to be 20—40 times that of the blood plasma (*cf* Lloyd and Sinclair 1953) The relatively high concentration of radioactivity in the lymphatic tissues which was noted in the present investigation seems to be consistent with these data In scurvy the phagocytosis of the mononuclear leucocytes is considered to be impaired (Follis 1958) Leblond (1934) has demonstrated a positive histochemical reaction for vitamin C in the mono nuclear leucocytes

The uptake in the bone marrow may be put in relation to the anemia associated with ascorbic acid deficiency (*cf* Lloyd and Sinclair 1953 Degk witz et al 1965)

The digestive system

The high concentration of ascorbic acid in the tissues of the alimentary tract does not seem to be related to a large excretion The concentration of ascorbic acid in the saliva has been found to be very low In humans it seems to be 5—10 times less than the concentration in the blood In the gastric secretion the concentration is about the same as in the blood (*cf* Lloyd and Sinclair 1953) The fecal excretion of ascorbic acid is very low and after an intraperitoneal injection of ascorbic acid 1 C^{14} to guinea pigs less than 1 per cent of the dose appeared in the faeces during 24 hours (Burns et al 1951)

After injection of labelled 3 4 dihydroxyphenylalanine to mice there was a considerable accumulation of radioactivity in the submaxillary salivary gland (Rosell et al 1963) The accumulation of labelled 5 hydroxytryptophan seems to be less pronounced (Ritzen et al 1965) By fluorescence microscopy has been shown that the submaxillary gland in the rat is richly supplied with adrenergic structures whereas the sublingual salivary gland seems to be devoid of adrenergic innervation (Norberg and Hamberger 1964 Norberg and Olson 1965)

The function of ascorbic acid in the gastro intestinal mucosa does not seem to be known Some of the radioactivity may be put in relation to the synthesis of serotonin in the enterochromaffin cells The distribution of radio activity however was rather even and no specific uptake in these cells could be observed

A positive histochemical reaction for vitamin C has been demonstrated in most cells of the mucosa of the human stomach and small intestine. Especially strong reaction has been obtained in the enterochromaffin cells (Clara 1943). In guinea pigs and rats these cells seem to contain less ascorbic acid as indicated by the histochemical studies (Pfuhl 1941, Clara 1954). In agreement with the present investigation the concentration of ascorbic acid in the cells of the mucosa of the large intestine has been found to be less than in the mucosa of the stomach and small intestine.

The respiratory system

A rather strong accumulation of radioactivity was observed in the bronchial and tracheal epithelium and also in the nasal mucosa. The localization may be put in relation to the monoamine containing cells in the tracheal epithelium which has recently been demonstrated (Larson et al. 1966).

The endocrine system

In ascorbic acid deficiency the adrenal gland has been found to hypertrophy and there is also a decrease in the concentration of cholesterol in the adrenal (Banerjee and Deb 1951, Eisenstein and Shank 1951). Stimulation of the adrenal gland with ACTH causes a decreased concentration of both ascorbic acid and cholesterol (Sayers et al. 1944). Since cholesterol is a precursor to steroid hormones, a role for ascorbic acid in the synthesis of some of these has been suggested. Several investigations indicate that ascorbic acid is concerned with the hydroxylation of some precursors to steroid hormones (cf. Degkwitz et al. 1965). So far no definite function has been established for ascorbic acid in the adrenal cortex.

There are several theories for the explanation of the decrease in ascorbic acid in the adrenal cortex during stimulation (cf. Pirani 1952, Lloyd and Sinclair 1953, Degkwitz et al. 1965). Ascorbic acid does not seem to be metabolized by the adrenal to substances no longer identifiable as ascorbic acid or dehydroascorbic acid (Salomon 1952).

In the present investigation dehydroascorbic acid-1-C^{14} had almost no affinity to the adrenal cortex. It is possible that ascorbic acid is oxidized at the reactions induced by ACTH in the adrenal cortex and then leaves the cells as dehydroascorbic acid. Dehydroascorbic acid has been shown to be rapidly taken up and reduced by the erythrocytes (Lloyd and Sinclair 1953). This may explain why Vogt (1948) found no increase in total ascorbic acid (ascorbic acid + dehydroascorbic acid) in venous blood plasma from the adrenal of dogs and cats after treatment with ACTH. This is also consistent with the results of Slusher and Roberts (1957) who noted an increase in the total ascorbic acid in the whole blood from the adrenal after ACTH stimulation.

According to earlier histochemical investigations the concentration of ascorbic acid in the adrenal cortex is high in both the zonae fasciculata and reticularis but lower in the zona glomerulosa (Leblond 1934 Clara 1954) This is in agreement with the distribution of radioactivity 4 hours after injection of ascorbic acid-1-C^{14} and dehydroascorbic acid-1-C^{14} Shortly after injection of ascorbic acid 1 C^{14} however the highest concentration in the adrenal cortex was found in the zona glomerulosa

In the adrenal medulla an even distribution of ascorbic acid has been demonstrated histochemically There has been great variations in the strength of the positive reactions and there has been doubt whether these are true reactions of vitamin C (Clara 1954) Both ascorbic acid and dehydroascorbic acid may be concerned with the synthesis of catecholamines and serotonin in the adrenal medulla Serotonin has recently been shown to be a physiological constituent of the adrenal medulla (Snyder et al 1965)

The concentration of ascorbic acid in the ovary is influenced by hormonal stimulation like that of the adrenal cortex (Claesson et al 1949 Hokfelt 1950) Also in the ovary ascorbic acid has been suggested to take part in the synthesis of steroid hormones It is therefore remarkable that there was not the same difference in the accumulation rate after injection of the labelled vitamin forms as was observed in the adrenal cortex The fact that there was a rather slow uptake of radioactivity after injection of either one of the two vitamin forms may be related to the advanced stage of pregnancy of the animals It has been shown that the corpora lutea contain considerably less ascorbic acid in late pregnancy than in the earlier stages (Giroud and Ratsimamanga 1942)

The distribution of radioactivity in the ovary showed great similarities with the distribution of injected C^{14} cholesterol (Appelgren in preparation)

Ascorbic acid seems to be of great importance for the carbohydrate metabolism Scorbutic guinea pigs are reported to have a reduced glucose tolerance (Sigal and King 1936) Their pancreatic islets are increased in number and size according to Banerjee (1944) but the cells contain few granules There is a decreased amount of insulin in the pancreas and a lowered deposition of liver glycogen (Banerjee and Ghosh 1947) Injection of dehydroascorbic acid induced a temporary or permanent diabetes (Patterson 1949 1950)

In the present investigation it is interesting to note the rapid uptake of radioactivity in pancreatic islets after injection of dehydroascorbic acid 1 C^{14} and the more slow accumulation of ascorbic acid 1 C^{14} Since the B cells constitute the major part of the pancreatic islet the autoradiographic distribution pictures indicate an accumulation at least in these cells An uptake also in other islet cells seems probable

Serotonin has been demonstrated in the pancreatic B cells of guinea pigs and mice (Falk and Hellman 1963 1964 Ritzen et al 1965) A metastazing

B cell adenoma in man has also been described which had an excessive production of both insulin and serotonin (Gloor et al 1964) In the duck a primary catecholamine has been demonstrated in the A cells (Cegrell et al 1964) After injection of H^3-labelled 3 4 dihydoxyphenylalanine to mice and rats there was a marked uptake of radioactivity in some few cells of the pancreatic islet possibly the A cells (Hempel 1963 Hammarström Johansson Ullberg in preparation)

The rapid uptake of dehydroascorbic acid 1-C^{14} and the presence of serotonin in the B cells of the pancreatic islet makes it likely that dehydroascorbic acid is involved in the synthesis of serotonin also in these cells This may suggest a role for serotonin in the induction of diabetes by dehydroascorbic acid Also the normal function of vitamin C in the synthesis and/or release of insulin may be mediated via dehydroascorbic acid and serotonin Ascorbic acid may also be implicated in the metabolism of the catecholamine containing cells of the pancreatic islets

In most species investigated histochemically there was a negative reaction for vitamin C unless parenteral administration of ascorbic acid preceded the investigation After administration of ascorbic acid almost all the cells of the pancreatic islet showed a positive reaction Some few cells possibly the A cells seemed to contain more ascorbic acid than the average (*cf* Clara 1934)

Alloxan which is chemically very similar to dehydroascorbic acid has recently been shown to be specifically taken up in the pancreatic islets of adult mice but not in the islets of young mice and rats (Hammarström and Ullberg 1966 Hammarstrom Hellman and Ullberg in preparation) Young animals have been found to be resistent to the diabetogenic action of alloxan (Ferner 1952) It is interesting to note that neither ascorbic acid dehydro ascorbic acid nor alloxan were specifically taken up in the pancreatic islets of young animals

In the thyroid the highest concentration was found in some few scattered cells probably the parafollicular cells These cells have been shown to contain serotonin (Falk et al 1964 Ritzen et al 1965) and possibly also a catecholamine (Larson et al 1966 Hammarstrom Johansson Ullberg in preparation) The function of these cells does not seem to be clarified There is some histochemical evidence that calcitonin is synthesized there (Foster et al 1964) By means of an antibody fluorescence technique however calcitonin has been demonstrated in the ordinary follicular cell of the thyroid (Hargis et al 1966)

The parathyroid accumulated radioactivity The function of ascorbic acid in this gland is not known but it may be put in relation to the accumulation of 3 4 dihydroxyphenylalanine H^3 (Hammarstrom Johansson Ullberg in preparation)

Connective tissue cartilage bone and teeth

The morphological changes associated with scurvy in connective tissue proper cartilage bone and teeth have been described in detail (*cf* Leicester 1949 Follis 1958) The basic biochemical defect in these tissues seems to be an impaired hydroxylation of proline and possibly also of lysine (Robertson 1964) In the present investigation there was a low concentration of radioactivity in the connective tissue proper This may be correlated to the slow rate of metabolism of this tissue and its low sensitivity to ascorbic acid deficiency (Neuberger and Slack 1953 Gould 1961 Robertson 1961)

In the odontoblasts there was a considerable uptake of radioactivity which seems to be in agreement with the fact that these cells are more sensitive to ascorbic acid deficiency than most other cells (Hojer 1926 Kuether et al 1944)

The odontoblasts fibroblasts and osteoblasts are considered to revert to an immature state in ascorbic acid deficiency (MacLean et al 1939 Thorell and Wilton 1945) The mechanism behind this maintenance of maturity by ascorbic acid seems to be obscure A considerable synthesis of collagen is going on in the odontoblasts as indicated by their uptake of labelled proline (Greulish and Slavkin 1965) Also in the other cells of the pulp the concentration of C^{14} ascorbic acid was higher than in the circulating blood This uptake does not seem to be due to an uptake in the walls of the blood vessels since no specific uptake in these could be observed elsewhere in the body In ascorbic acid deficiency the dental pulp becomes hemorrhagic and a deposition of an amorphous calcified material occurs (Höjer and Westin 1925 Boyle et al 1940)

In cartilage and bone of the young animals the concentration of C^{14} ascorbic acid was higher than in the proper connective tissue The autoradiographic localization of ascorbic acid in these tissues is in agreement with earlier histochemical studies showing a markedly positive reaction in the zone of cartilage cell division and a negative reaction in the hypertrophied cartilage cells In the calcifying cartilage and in the newly formed bone trabeculae the reaction was positive again (Bourne 1956) Also the uptake at the predentin dentin junction has earlier been demonstrated with histochemical methods (Naujoks 1956 Harndt 1960) The persisting localization of ascorbic acid in these areas may indicate a role in the physiological mineralization process In scurvy the calcified cartilage in the epiphyseal plate is claimed not to be replaced by bone trabeculae and the predentin becomes hypercalcified (*cf* Leicester 1949 Follis 1958)

In the enamel of some teeth there was a rapid and pronounced uptake of C^{14} *ascorbic acid* Also here the radioactivity was localized in areas where mineralization apparently was taking place as indicated by the distribution of injected radiocalcium in the teeth of rats of the same age (Fig 56) With



Fig 56 Microautoradiogram (section + autoradiogram) of the first upper molar of a 10 days old rat 30 minutes after an i p injection of $Ca^{45}Cl$ There is a high concentration (black grains) throughout the thickness of the enamel at the tip of the cusps and a less marked uptake in the superficial parts of the enamel matrix underneath the tall ameloblasts in the cervical parts of the tooth There is also an accumulation in the dentin close to the pulp There seems to be no radioactivity on the dentin side of the amelodentinal junction in the zones of secondary mineralization of the enamel as was seen after injection of ascorbic acid 1 C^{14} (Hammarstrom, unpublished) (x 40)

time the C^{14} ascorbic acid seemed to be redistributed to areas where mineralization was going on at the moment

Ascorbic acid deficiency is considered to have no direct effect upon enamel formation (Boyle 1938 Wassermann 1944 Fullmer et al 1961) The dental changes associated with scurvy however have mainly been studied using decalcified histological sections and possible changes in the composition might not have been detected

It has been shown that during the mineralization of the enamel there is a considerable loss of organic material of the matrix once deposited There is at least an 80 per cent (w/v) decrease in the concentration of all amino acids except hydroxyproline and hydroxylysine (Deakins 1942 Weinmann

et al 1942 Eastoe 1963 Burgess and Maclaren 1965) In fact there seen to be some increase in the amount of hydroxyproline (Burgess and Maclare 1965) The major changes in the composition of the organic components (the enamel matrix apparently take place immediately after the matr reaching its final thickness Thi tate of development seems to be identic with the one of rapid mineralization and uptake of ascorbic acid No signif cant amounts of hydroxyproline and hydroxylysine have been demonstrat in fetal enamel (Eastoe 1960 Glimcher et al 1961 Piez 1961) In matu enamel on the other hand several investigators have demonstrated both hydr xyproline and hydroxylysine (Hess et al 1953 Stack 1954 Battistone a Burnett 1956 Burgess and Maclaren 1965) The amounts are small a contamination may easily occur both from the dentin and from the saliva a a possible coronal cementum (Glimcher et al 1964) It is however tempti to assume that ascorbic acid is involved in the hydroxylation of proline a lysine also in the enamel The fact that only the reduced form was accum lated in the enamel ma support this suggestion The hydroxylation m be of importance for a siolog cal deposition minerals During scurvy amorphous calcification curs in arious tissues (Bourne 1956)

There are some rec ts of an impaired calcium metabolism in scur But the changes have been c nsidered to be secondary to an imperfect mat formation of bone and ten in (Bourne 1943 1956)

The finding by Toverud (1923) of a marked decrease in total ash a calcium in teeth of scorbutic guinea pigs is interesting in view of the possi role of ascorbic cid in the mineralization process Friberg (1958) notec decreased uptake of labelled phosphate in bone and teeth of scorbutic gui pigs

The autoradiographic pictures of Ca[4] and C^{14}-ascorbic acid in the ena support the idea of a second mineralization starting in the cusps when partly mineralized matrix has reached its final thickness (Diamond and We mann 1940 Glock et al 1942 and others)

The au oradiographic blackening in the dentin close to the amelodent junction apparently has no corresponding accumulation of minerals (Fig : Presently no specific chemical reaction is known to take place in those a of the dentin in which ascorbic acid might take part But it indicate possible biochemical interrelationship between reactions in the enamel : dentin of the developing teeth

The distribution of radioactivity in the periodontal tissues may indi that the loosening of teeth in scurvy is more a matter of decreased fixa of the periodontal fibers in the bone than an impaired maintenance of fibers themselves This is in agreement with earlier investigations show a marked resorption of the alveolar bone associated with ascorbic : deficiency (Wagner 1932 Boyle 1941 Shaw et al 1945)

SUMMARY

Ascorbic acid 1 C^{14} and dehydroascorbic acid 1 C^{14} have been administered intravenously to adult male mice pregnant female mice and 16 days old rats and intraperitoneally to 10 days old rats The distribution of the injected substances at various intervals (2 minutes—6 days) after injection has been studied by whole body autoradiography microautoradiography and impulse counting The chemical identity of the radioactivity in the tissues at various intervals (5 minutes—4 days) after injection has been investigated by thin layer chromatography of extracts of some selected tissues which showed a high concentration of radioactivity

The chromatographic investigation indicated that the radioactivity in the adrenals liver spleen kidney salivary glands and brain almost exclusively represented ascorbic acid regardless of whether ascorbic acid 1 C^{14} or dehydroascorbic acid 1 C^{14} had been injected In spite of the rapid reduction of injected C^{14} dehydroascorbic acid the distribution of the two vitamin forms were very different after intravenous injection The distribution patterns gradually became similar but as long as 3—4 days after injection some differences were noticeable The distribution patterns have been described and discussed in detail The results which functionally seem to be most interesting have been summarized below

After an intravenous injection of C^{14}-ascorbic acid the accumulation in the central nervous system proceeded very slowly There seemed to be a complete blood brain barrier and the radioactivity penetrated from the choroid plexa and from the periphery possibly together with simultaneously secreted cerebrospinal fluid At [illegible] days after injection the central nervous system showed the highest concentration in the body

After an intravenous injection of C^{14} dehydroascorbic acid the concentration in the central nervous system rapidly increased and as early as 2 minutes after injection radioactivity was found in the whole brain and spinal cord and the concentration markedly exceeded that in the blood The striking difference in rate of penetration was also demonstrated by the impulse counting investigation After as long as 4 days the concentration in the brain was higher after injection of C^{14} dehydroascorbic acid than after C^{14} ascorbic acid

Injection of dehydroascorbic acid has been found to cause an intense hyperactivity of short duration (Patterson and Mastin 1951) and also a rise in the higher nervous activity lasting for several days (Lyhs and Tegeler 1963) This may be put in relation to the rapid and persistant accumulation of

et al 1942 Eastoe 1963 Burgess and Maclaren 1965) In fact there seems to be some increase in the amount of hydroxyproline (Burgess and Maclaren 1965) The major changes in the composition of the organic components of the enamel matrix apparently take place immediately after the matrix reaching its final thickness This tate of development seems to be identical with the one of rapid mineralization and uptake of ascorbic acid No significant amounts of hydroxyproline and hydroxylysine have been demonstrated in fetal enamel (Eastoe 1960 Glimcher et al 1961 Piez 1961) In mature enamel on the other hand several investigators have demonstrated both hydroxyproline and hydroxylysine (Hess et al 1953 Stack 1954 Battistone and Burnett 1956 Burgess and Maclaren 1965) The amounts are small and contamination may easily occur both from the dentin and from the saliva and a possible coronal cementum (Glimcher et al 1964) It is however tempting to assume that ascorbic acid is involved in the hydroxylation of proline and lysine also in the enamel The fact that only the reduced form was accumulated in the enamel may support this suggestion The hydroxylation may be of importance for a [illegible]ysiolo[illegible]al deposition minerals During scurvy an amorphous calcification [illegible]curs in [illegible]ious tissues (Bourne 1956)

There are some re[illegible] of [illegible] impaired calcium metabolism in scurvy But the changes have [illegible] to be secondary to an imperfect matrix formation of bone an[illegible] (Bourne 1943 1956)

The finding by [illegible] (23) of a marked decrease in total ash and calcium in teeth of [illegible]inea pigs is interesting in view of the possible role of ascorbic [illegible]mineralization process Friberg (1958) noted a decreased uptake [illegible] phosphate in bone and teeth of scorbutic guinea pigs

The autoradiograph[illegible] of Ca^{45} and C^{14} ascorbic acid in the enamel support the idea of [illegible] mineralization starting in the cusps when the partly mineralized mat[illegible] reached its final thickness (Diamond and Weinmann 1940 Clock et al 1942 and others)

The autoradiographic blackening in the dentin close to the amelodentinal junction apparently has no corresponding accumulation of minerals (Fig 56) Presently no specific chemical reaction is known to take place in those areas of the dentin in which ascorbic acid might take part But it indicates a possible biochemical interrelationship between reactions in the enamel and dentin of the developing teeth

The distribution of radioactivity in the periodontal tissues may indicate that the loosening of teeth in scurvy is more a matter of decreased fixation of the periodontal fibers in the bone than an impaired maintenance of the fibers themselves This is in agreement with earlier investigations showing a marked resorption of the alveolar bone associated with ascorbic acid deficiency (Wagner 1932 Boyle 1941 Shaw et al 1945)

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CONTENTS

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INTRODUCTION

The preaortal paraganglia (or Zuckerkandl organs) constitute large sources of catecholamines in fetal and newborn mammals The physiological role of these organs has however not yet been clarified — In the present work the preaortal paraganglia of newborn rabbits have been investigated with regard to their storage synthesis and release of catecholamines Most experimental series have been performed as comparative studies using the adrenal glands of the same animals as reference organs

CHAPTER I

HISTORICAL SURVEY

Chromaffin tissue containing catecholamines (adrenaline noradrenaline and in some species dopamine) has been found in all mammalian species studied (Euler 1956) Owing to the important biological effects of the catecholamines for maintaining a physiological homeostasis especially in stressful situations the localization and functions of the chromaffin tissues have been extensively studied (for ref see West 1955 Coupland 1965) Chromaffin cells have thus been found partly as small groups of cells scattered in various organs throughout the body and partly in the form of a highly specialized organ the adrenal medulla In adult animals this organ constitutes the main source of catecholamines

In fetal and newborn mammals however an additional type of chromaffin tissue has been identified in the vicinity of the adrenal glands Dostoiewsky (1886) originally demonstrated the presence of this extra adrenal chromaffin tissue in several mammalian species describing it as accessorische primitive Nebennieren It was not until 1901 however that Zuckerkandl was able to demonstrate that this tissue in fetal and newborn humans, besides occurring as small islets of chromaffin cells around the adrenal glands also constitutes a well defined paired retroperitoneal organ on the ventral wall of the abdominal aorta In subsequent literature these organs have frequently been referred to as Zuckerkandl organs Kohn (1903) performed the first detailed comparative study on the anatomical relationships between the adrenal medulla and the paraganglion aorticum abdominale or paraganglia , as he named the extra adrenal chromaffin tissue Kohn showed that the embryonic development and final anatomical arrangement of the paraganglia are different in different mammalian species Thus in man and cat the paraganglia are formed by ectodermal cells migrating forward from the neural crest to both sides of the aorta where they form large clusters of chromaffin cells surrounded by smaller islets of similar cells In these species there is no direct anatomical continuity between the chromaffin cells of the paraganglia and those of the adrenal medulla In dogs an almost identical development

was described by Kahn (1912), whereas in rabbits as shown by Kohn (1903) both the paraganglionic and adrenomedullary cells develop within one and the same morphological unit Thus the abdominal chromaffin cells in the rabbit embryo form two strands of cells one on each side of the abdominal aorta At about 15 days of gestation the cranial portions of these strands become partly enclosed by the adrenocortical germ cells The caudal portions melt together onto the ventral wall of the aorta into one slightly bipartite mass of chromaffin cells which then constitutes the paraganglion The direct anatomical connection between the paraganglion and the adrenal medullae of both sides persists even some time after birth — As compared to those other mammalian species studied the paraganglionic cells of the rabbit are considerably more confined to the large preaortal cell masses the surrounding groups of accessory chromaffin cells being few and small

In humans the paraganglia undergo fibrotic degeneration soon after birth, and at the age of 3 to 5 years connective tissue occupies almost the entire organ (Zuckerkandl 1901 1911) However the information on the postnatal fate of the paraganglia in other mammals is to some extent controversial According to Kohn (1903) the paraganglia of cats and rabbits persist throughout life (*cf* Coupland 1956) On the other hand Pellegrini (1906) found degenerative changes in the paraganglionic tissue of several mammals dog and mouse as well as cat and rabbit In rats an almost total degeneration of the paraganglia occurs soon after birth (Coupland 1960 and others) Recently Lempinen (1964) reported that this postnatal involution of the rat s paraganglia could be prevented or inhibited by the administration of glucocorticoid hormones

On the functional role of the paraganglia our information is very limited Biedl and Wiesel (1902) showed that extracts of paraganglia from newborn humans contained substances with pressor effects similar to those previously obtained with adrenal extracts (Oliver and Schafer 1895) Paraganglia from cats and dogs were also found to contain pressor substances (Vincent 1910 Kahn 1912 and others) Lelkes (1941) using a bioassay technique observed that in the same human *foetus* the amounts of pressor substances in the paraganglia exceeded those found in the adrenal glands Similar quantitative results have been reported by several authors (for ref see West *et al* 1953) Separate determinations of adrenaline and noradrenaline revealed that the pressor substances almost exclusively consisted of noradrenaline in the paraganglia of all the mammalian species studied man dog cat rabbit and guinea pig (Shepherd and West 1952, Nemineva and Pekkarinen 1952 and others) Traces of adrenaline were also found in the paraganglia of certain

specimens No precursors or metabolites of catecholamines were demonstrated in the paraganglia

In the adrenal glands of fetal and newborn animals the ratio of noradrenaline/adrenaline was found to be higher than in the adult (West and Hunter 1951, Holtz 1951 and others) Shepherd and West (1951) reported that in the adrenal glands of fetal rabbits no adrenaline could be recovered and that the total catecholamine content consisted of noradrenaline However recently two independent studies on the adrenal catecholamines of the developing rabbit *foetus* showed that after 24 days of gestation the adrenaline content far exceeded that of noradrenaline (Roffi 1964, Brundin 1965 a)

The subcellular organization of the catecholamine storing organs has become the object for extensive studies in recent years It is now well established that in the adrenomedullary cells as well as in sympathetic nerve endings the catecholamines are bound to cytoplasmic osmiophilic granules visible in the electron microscope (Hillarp Lagerstedt and Nilson 1953 Blaschko and Welch 1953, Lever 1955, Euler and Hillarp 1956 Lever and Esterhuizen 1961) It has also been described that the granules in adrenomedullary cells differ from nerve granules with respect to both their catecholamine binding properties and their reactions to certain pharmacological agents (for ref see Stjarne 1964) Concerning the subcellular organization of the paraganglionic tissue however no information seems to be available either with regard to its mechanisms for catecholamine storage or to its ultrastructure in general

Since Gurin and Delluva (1947) were able to isolate C^{14} labeled adrenaline from the adrenal glands of rats pretreated with phenylalanine C^{14} the biosynthesis of catecholamines in the adrenal medulla has been intensely studied In experiments using both *in vivo* and *in vitro* techniques the pathway of catecholamine formation from tyrosine via dihydroxyphenylalanine and dopamine previously outlined by Blaschko (1939) has been confirmed by several investigators (for ref see Kirshner 1958 Udenfriend 1965) After studies on the different steps of the synthetic pathway the conversion of tyrosine to dihydroxyphenylalanine was found to be a time consuming process whereas the subsequent steps decarboxylation to dopamine hydroxylation to noradrenaline and methylation to adrenaline have been regarded as rapid reactions (Rosenfeld Leeper and Udenfriend 1958) — There is no information available regarding the capacity of noradrenaline synthesis in the paraganglia Nor is it known if the paraganglia possess any mechanisms for selective uptake of noradrenaline In the adrenal medulla the uptake of injected adrenaline has been found to be low (Udenfriend and Wyngaarden 1956)

It is well known that the release of catecholamines from the adrenal medulla into the blood stream can be elicited by stimulation of the splanchnic nerves

(Dreyer 1899) Thus the dramatic increase of adrenaline release during hypoglycemic conditions seems to be mediated exclusively by this innervation (for ref see Duner 1953) On the other hand certain patho physiological conditions such as asphyxia and hypoxia have been found to cause increased release of catecholamines also from denervated adrenal glands (Comline and Silver 1958 1961, Comline Silver and Silver 1963) Recently this non nervous or direct stimulation of the adrenal medulla has been found more easily demonstrable in fetal and newborn animals than in the adult (Comline and Silver 1966) — The question whether or not the paraganglionic tissue is furnished with any functional innervation has not yet been definitely clarified In morpological studies on dogs and cats Hollinshead (1937) has described nerve fibres with terminals in the paraganglionic tissue Since no such nerve fibers could be observed after section of the *rami communicantes* of the lumbar segments it was assumed that the paraganglia had a preganglionic innervation similar to that of the adrenal medulla However by means of electrical stimulation of the inferior splanchnic nerves of young dogs no support was obtained for any functional preganglionic innervation of the paraganglionic tissue (Muscholl and Vogt 1964)

The paraganglia are supplied with a dense vascular net (Kohn 1903 and others) Therefore substances released from the paraganglionic cells into the blood could easily be distributed in the organism However it is not yet known whether or not the paraganglia are able to release catecholamines under physiological or patho physiological conditions

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The following abbreviations have been used

A = adrenaline
CA = catecholamine(s)
DA = dopamine
DOPA = dihydroxyphenylalanine
NA = noradrenaline
f u = fluorescence units

CHAPTER II

GENERAL METHODS

Material

Albino rabbits were used For good access of fetal and newborn animals a rabbit breeding was organized The rabbits were mated under observation The time of gestation was regularly 30—31 days (*cf* Asdell 1946) The deliveries observed seldom exceeded 4 hrs Most litters contained 6—8 youngs of 35—55 g each When the newborn rabbits were kept apart from their mothers in the nest at room temperature, their body temperature (intracolic) was relatively constant, 34 0—36 5°C In those experiments where an experimental group of animals has been compared to a control group both groups have been recruited from the same litter

All the experiments were performed from March to October During the winter no experiments were carried out because only few youngs were obtained from the breeding at this time of the year when many doe rabbits where in anestrus Pseudopregnancies also occurred frequently during the winter

Technique of dissection

In the studies on *foetus* the doe rabbit was killed by a blow on the head and the *foetus* were immediately removed and decapitated The newborn animals were killed by decapitation Through a large abdominal incision the preaortal paraganglion was carefully dissected from the adjacent tissues under a dissection microscope The paraganglion was separated from the adrenal glands as shown in Fig 1 The thin cranial section of the paraganglion extending to the right adrenal gland was left in the animal since it was covered by liver tissue and impossible to excise accurately In those experiments where the adrenal glands were also studied these were dissected after the removal of the paraganglia

Organ weights

The paraganglia and adrenal glands from one litter of 7 newborn rabbits were weighed on a balance (Mettler type H 16) with a sensitivity of ± 0 02 mg

During the short interval between dissection and weighing the organs were kept in an ice cold metal bowl in order to reduce weight loss caused by evaporation The mean weight of the paraganglia was 2 9 mg with a range of 2 0—5 3 mg The adrenal pairs (right and left) had a mean weight of 6 9 mg with a range of 2 7—9 6 mg

Because of this wide variation in organ weights all CA values are expressed as μg per paraganglion or per pair of adrenal glands

Extraction of catecholamines

Immediately after the excision the paraganglia and adrenal glands were ground separately with chemically pure quartz sand in 2 ml of 0 4 M per

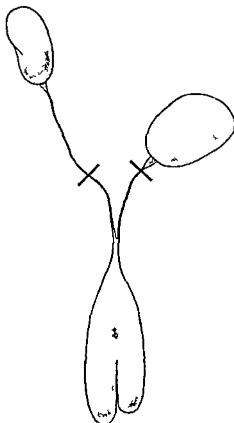


Fig 1 Paraganglion and adrenal glands from a newborn rabbit Double lines indicate the levels where the paraganglion was cut and separated from the adrenal glands

In one case the paraganglion was found aberrant being divided into cranial and caudal halves separated by a diastasis of about 2 mm

chloric acid. Unless stated otherwise the paraganglia were extracted one by one, the adrenal glands by pairs. The extracts were stored at —20°C until analysis. When stored more than one week or when the CA of the extracts were to be separated chromatographically the extracts were adjusted to pH 4 (*cf.* Gunne 1963) by titration with 2 N potassium hydroxide and centrifuged at 15,000 × *g* and 0°C for 10 min to remove precipitating potassium per chlorate. The extracts were thoroughly stirred during thawing in order to avoid separation caused by cryoscopic effects (Rangappa 1961).

Fluorimetric determinations of noradrenaline and adrenaline

The concentrations of NA and A in the extracts were determined photo fluorimetrically using the trihydroxyindole method (Ehrlen 1948, Lund 1949, Euler and Lishajko 1959) in which the CA are oxidized and rearranged to their respective lutines, the fluorescence of which is measured.

Due to the high concentrations of CA in the actual extracts they could be assayed directly without any preceding purification procedure. After centri fugation at 800 × *g* at room temperature for 5 min, 0.5 ml of the extracts was adjusted to pH 6.5 by addition of 2.5 ml 0.25 M sodium phosphate buffer and oxidized with 0.1 ml 0.25 % potassium ferricyanide solution for 3 min. The oxidation was interrupted with 4 ml 0.2 % ascorbic acid in 5 N sodium hydroxide. Ethylene diamine was added in order to stabilize the fluorescence according to Euler and Lishajko (1961). The fluorescence was measured in a photofluorometer (Turner model 110) with 2 sets of filter combinations (Schott 395 nm/Ilford Bright 490 nm and Schott 436 nm/Corning 540 nm). The mean recovery for added NA and A was 97 % with a range of 94—101 % in several control tests. The NA and A values are expressed as the bases of the respective amines.

Separation of catecholamines

Separation of NA, A and DA was performed by means of an ion exchange chromatographic technique according to Häggendal (1962). A strong cation exchange resin (Amberlite CG 120, type 2, mesh 200—400) was used in Na form. Separation of NA, A and DA was performed on resin columns of 15×0.4 cm. When only NA and DA were to be separated 5×0.4 cm columns were used. The organ extracts were of about 3 ml and adjusted to pH 4. After adsorption of the extract the column was washed with 3 ml 1 M sodium acetate buffer pH 4 and 3 ml redistilled water. A pressure of 100 mm Hg was applied during the adsorption and rinsing processes but not during the

subsequent elution The elution of NA and A from the 15 cm columns was performed with 0 4 N hydrochloric acid DA was then eluted with 2 N hydro chloric acid from the same columns From the 5 cm columns NA and DA were eluted with 1 N hydrochloric acid To protect the amines from oxida tion in the columns ascorbic acid, 10 µg/ml was added to the eluant The eluate was separated into 1 1 ml fractions by a fraction collector with drop counter (LKB Radi Rac) The direct fluorescence of each fraction was measured at 285/335 nm wavelengths (activ /fluor instrumental values) in an Aminco Bowman spectrophotofluorometer and compared to a NA standard solution (1 µg NA/ml = 50 f u) By this method the following recovery values were obtained

15 cm columns, NA and A 70—80% DA 35—55 %
5 cm columns NA 80—90 % DA 35—45 %

Radioactivity measurement

In certain experiments the radioactivity was measured in the fractions of the chromatograms A liquid scintillation technique was employed Sample vo lumes of 500 µl were diluted with 6 ml absolute ethanol and 14 ml toluene containing 4 g/l of 2 5 diphenyloxazole and 0 10 g/l of 1 4 bis 2(5 phenyl oxazolyl) benzene and counted for 10 min in a liquid scintillation spectro meter (Packard Tri Carb) Several series of samples were counted in dupli cates and showed good agreement even with activities below 100 cpm The radioactivity of each fraction in the chromatograms is expressed as cpm The background radioactivity never exceeded 750 counts when repeatedly con troled by counting blank samples for 10 min on the C^{14} channel The instru mental background level was therefore set to 75 cpm

CHAPTER III

STUDIES ON THE CATECHOLAMINE STORES IN THE PARAGANGLIA

Special methods

Identification of catecholamine in paraganglia

In extracts of pooled paraganglia from newborn rabbits NA A and DA were analyzed after separation on 15 cm Amberlite columns After measurement of the direct fluorescence of each fraction the fractions at the positions of NA A and DA were adjusted to pH 6 5 by the addition of 0 5 M potassium phosphate buffer The NA and A fractions were then oxidized with potassium ferricyanide and the DA fractions were oxidized with iodine (*cf* Euler and Lishajko 1959 1961 Carlsson and Waldeck 1958) The fluorescence of the oxidation products was read at 410/535 nm for NA at 425/545 nm for A and at 345/410 nm for DA (uncorrected instrumental wavelength values) in an Aminco Bowman spectrophotofluorometer

Corresponding chromatographic separations were performed on extracts of the paraganglion and the adrenal glands from a one day old rabbit which had received *dl* dihydroxyphenylalanine 2 C^{14}, 0 12 μC/g b w s c 12 hrs before the dissection A and DA were added to the paraganglion extract before the separation The CA were localized in the chromatograms by measuring the direct fluorescence of each fraction The radioactivity of each fraction was measured by liquid scintillation

Histochemical studies*

In order to study the localization of CA in the paraganglia at different stages of postnatal development a histochemical study was undertaken A total of

* These studies were performed in collaboration with B Hamberger and K A Norberg at the Department of Histology Karolinska Institutet

14 paraganglia from newborn and young rabbits were treated according to the formaldehyde condensation method of Falck and Hillarp (Falck 1962, Falck *et al.* 1962). By this method CA are condensed with formaldehyde to intensely fluorescent products. The chemical basis for the reaction and its specificity and sensitivity when used in tissues has been described and discussed by Corrodi and Hillarp (1963, 1964). For technical procedures see Norberg and Hamberger (1964), Falck and Owman (1965).

Differential centrifugation of homogenates of paraganglia and adrenal glands

Three experiments were performed on organs from 15 newborn rabbits. In each experiment 5 pooled paraganglia and 10 pooled adrenal glands were used. After the excision the organs were immediately immersed in 10 ml ice cold 0.13 M potassium phosphate buffer pH 7.4. The subsequent preparation was performed at +4°C. The pooled organs were then homogenized by grinding with celite (mesh 30—80). The homogenates were centrifuged at $1\,000 \times g$ for 10 min to remove coarse particles. From the approximate 10 ml of low speed supernatants obtained, 2 ml aliquots were centrifuged at $50\,000 \times g$ for 30 min in a refrigerated centrifuge (MSE Super Speed 25). The high speed supernatants were decanted in 0.5 ml 0.4 M perchloric acid and adjusted to pH 3.5 by titration with 2 M potassium hydroxide. Each high speed sediment was extracted with 1 ml 0.4 M perchloric acid. The CA contents of the extracts were determined fluorimetrically.

Electron microscopical studies*

Paraganglia and adrenal glands from 5 newborn rabbits were studied by electron microscopic technique. Under light ether anaesthesia fixation of the organs was instigated *in situ* by dripping the fixative onto the exposed organs for 5 min. The fixative used was a cold 1 % solution of osmium tetroxide buffered with Veronal acetate to pH 7.2—7.4 and made approximately isotonic to mammalian plasma. After excision of the organs the fixation was continued *in vitro* for 3 hrs. Rinsing in buffer solution and slow dehydration in acetone preceded embedding in Vestopal W. The sections were examined in an RCA EMU 3A electron microscope.

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* These studies were performed in collaboration with S. E. G. Nilsson at the Department of Anatomy, Karolinska Institutet.

Results

Identification of catecholamines in paraganglia

In extracts of 2, 2 and 3 paraganglia from 7 newborn rabbits the contents of NA, A and DA were measured after chromatographic separation. The chromatograms (Fig. 2) show that the direct fluorescence reached high values in the fractions at the position of NA. No other peaks were observed.

After oxidation, high fluorescence values at the maximum wavelengths for NA (410/535 nm) occurred in the same fractions which gave the direct fluorescence peaks. In the subsequent fractions, corresponding to the position of A, the fluorescence at the maximum wavelengths for A (425/545 nm) gave blank values. Neither was there any measurable fluorescence at the maximum wavelengths for DA (345/410 nm) in those fractions where this amine would be expected.

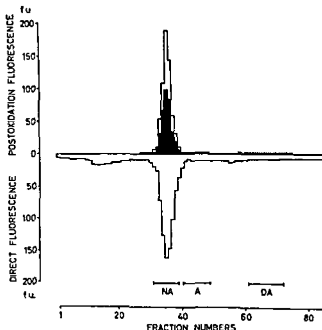


Fig. 2. Chromatographic distribution of direct fluorescence at 285/335 nm and postoxidation fluorescence at 410/535 nm □, 425/545 nm ■ and 345/410 nm after separation of CA in an extract of 2 pooled paraganglia of newborn rabbits. Fraction collection started at the adsorption of the extract on the column. Horizontal bars indicate the expected positions for NA, A and DA. Column length 15 cm.

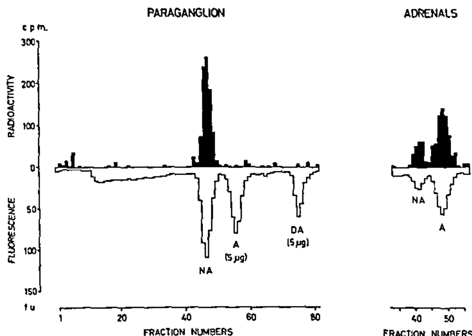


Fig 3 Chromatographic distribution of radioactivity and direct fluorescence after separation of CA in extracts of paraganglion and adrenal glands from a 1 day old rabbit 12 hrs after administration of DOPA C^{14} Adrenaline (A) and dopamine (DA) added to paraganglion extract Fraction collections started at the adsorption of extracts on the columns Column length 15 cm

In order to detect even trace amounts of A and DA the radioactivity of each fraction was measured after an identical chromatographic separation performed on a paraganglion extract from a one day old rabbit pretreated with a radioactively labeled CA precursor, DOPA C^{14} Unlabeled A and DA were added to the extract before the separation

A peak of radioactivity coincided with the direct fluorescence at the position of NA (Fig 3) while barely measurable traces of radioactivity were found in the A and DA fractions of the paraganglionic chromatogram However in the chromatogram of the adrenal glands from the same animal, peaks of radioactivity were obtained both in the NA and A fractions A C^{14} being quantitatively dominant

Table I CA contents in paraganglia (µg/organ) and adrenal glands (µg/pair of organs) from one litter of 6 fetal rabbits at 29 days of gestation

Foetus nr	PARAGANGLIA NA	ADRENALS NA	ADRENALS A
1	4.72	0.71	2.18
2	6.13	0.34	2.35
3	4.69	0.28	1.10
4	4.03	0.53	1.81
5	2.21	0.49	1.55
6	5.22	0.73	1.71
Mean ± S.E.M.	4.50 ± 0.3[illegible]	0.51 ± 0.07	1.[illegible]9 ± 0.19

Noradrenaline amounts in paraganglia from rabbits of different perinatal ages

The NA contents were measured fluorimetrically in the paraganglia from a total of 6 fetal and 32 young rabbits, 1 day to 4 week old. In the fetal series the contents of CA in the adrenal glands were also measured for comparison. The results are listed in Tables I—III in terms of µg/animal.

The *foetus* were removed from the uterus 29 days after mating (day of mating not included). The mean CA content in the paraganglia was about twice that of both the adrenal glands from the same *foetus* (Table I). The NA values for the fetal organs showed small variations with one exception: *foetus* nr 5.

Table II Contents of NA (µg per organ) in paraganglia from 5 litters (a—e) of 24 rabbits 1—7 days of age

	1 DAY		2 DAYS		5 DAYS			7 DAYS		
Litter	Animal nr	NA	Animal nr	NA	Litter	Animal nr	NA	Litter	Animal nr	NA
	1	6.72	4	7.44		13	8.4[illegible]		19	6.74
a	2	6.38	5	6.77		14	6.98	d	20	10.0
	3	6.[illegible]0	6	7.10		15	6.11		21	10.4
	7	7.[illegible]1	10	7.73	c	16	6.61		2[illegible]	3.[illegible]0
b	8	5.94	11	6.98		17	7.78	e	23	5.60
	9	6.38	1[illegible]	7.20		18	6.61		24	8.43
Mean ± S.E.M.		6.47 ± 0.18		7.[illegible]0 ± 0.14			7.09 ± 0.35			7.4[illegible] ± 1.1

Table III NA in paraganglia (µg per organ) from 2 litters of rabbits 2 and 4 weeks of age

2 WEEKS (litter f)		4 WEEKS (litter g)	
Animal nr	NA	Animal nr	NA
25	4.37	28	4.61
26	13.0	29	4.11
27	5.5	30	2.32
		31	5.20
		32	2.43
		Mean ± S.E.M.	3.74 ± 0.58

The NA contents in the organs from 1 day old rabbits (Table II) far exceeded those in the fetal organs and in the 2 and 5 day old rabbits the values were further increased. In the paraganglia of 7 day old animals the mean content of NA was essentially unchanged, but the individual values showed considerably wide variations. In two of the 2 week old rabbits (Table III) the NA

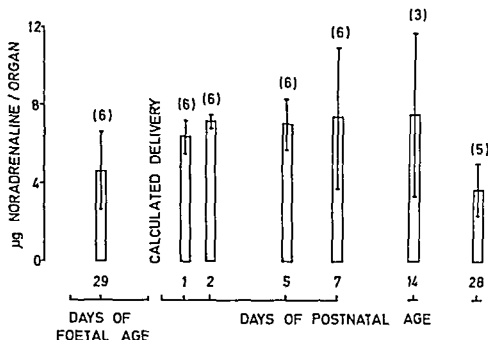


Fig. 4 Total contents of NA in paraganglia from rabbits at different stages of perinatal development. Mean and range values.

values were slightly reduced but in one specimen a remarkably high value was obtained After 4 weeks there was a definite decrease in the NA content the mean value being even lower than in the fetal organs

Fig 4 gives a summary of the mean contents of NA in the paraganglia during the perinatal period studied

Histochemical observations*

In order to find out whether the localization of CA in the paraganglia changed during the postnatal period of decreasing NA content in the organs a histochemical study was performed on paraganglia from newborn and young rabbits

In newborn rabbits (Fig 5) the paraganglia consisted of strands of polygonal or ovoid cells surrounded by a fibrous capsule The organ was divided by the capsule into two parts (*cf* Chapter I) The cytoplasm of the parenchymatous cells exhibited an intense yellow green fluorescence corresponding to high concentrations of CA This intense fluorescence was observed in all the cells throughout the parenchyma

Essentially the same pictures were obtained from paraganglia sections of rabbits two week old but some clusters of cells with lower fluorescence intensitie were also observed suggesting that these cells had partially lost their CA contents

In rabbits two month old the paraganglia were no longer enclosed by proper capsules Connective and to some extent, fatty tissue occupied almost the entire organ Only small islets of intensely fluorescent cells remained (Fig 6)

Distribution of catecholamines in sediments and supernatants after high speed centrifugation of homogenates of paraganglia and adrenal glands

The subcellular distribution of CA in paraganglia and adrenal glands of newborn rabbits was studied by differential centrifugation of organ homogenates The amounts of CA recovered from the sediments and supernatants obtained by high speed centrifugation are listed in Table IV In the paraganglionic homogenates 68—76 % of the total CA contents were recovered from the high speed sediments In the homogenates of the adrenal glands from the same animals 76—83 % of the total CA contents were bound to

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* A preliminary report of these observations has been published (Brundin Hamberger and Norberg 1966)

Table IV CA (μg) in high speed sediments (sed) and supernatants (su) from homogenates of pooled paraganglia and adrenals of newborn rabbits

Exp nr	NA in paraganglia homogenates				NA + A in adrenal homogenates			
	sed	su	total	sed/total %	sed	su	total	sed/total %
I	4.78	1.76	6.54	73	2.74	0.76	3.50	78
II	5.88	2.77	8.65	68	3.03	0.96	3.99	76
III	6.74	2.13	8.87	76	4.01	0.81	4.82	83

the sedimentable fraction. Thus the sedimentable portions of CA in the adrenal homogenates slightly exceeded those of the paraganglionic homogenates. This difference was however not statistically significant.

Electron microscopical observations*

In cells of paraganglia as well as of adrenal medulla from newborn rabbits large amounts of membrane limited osmiophilic granules were found (Fig 7—9). The adrenomedullary granule consisted of a relatively large electron dense core and a less electron dense dotted periphery. The paraganglionic granule exhibited a considerably smaller electron dense core and a larger peripheral zone of low opacity. These differences in appearance between the granules from the two organs were consistently observed in the sections from all the animals studied. However, the granules of both organs were of about the same size, their average diameters being about 0.15 μ.

Discussion and conclusions

The CA stores in paraganglia from newborn rabbits were found to consist of NA exclusively. This amine was identified in extracts of pooled organs by different methods, all giving congruent results. No A or DA was recovered from the extracts.

In earlier investigations of the CA content in paraganglionic tissues from several mammalian species small amounts of A have been found in some specimens (Shepherd and West 1952, Niemineva and Pekkarinen 1952, Greenberg and Lind 1961 and others). Also in two preliminary reports (Brundin

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* A preliminary report of these observations has been published (Brundin and Nilsson 1965

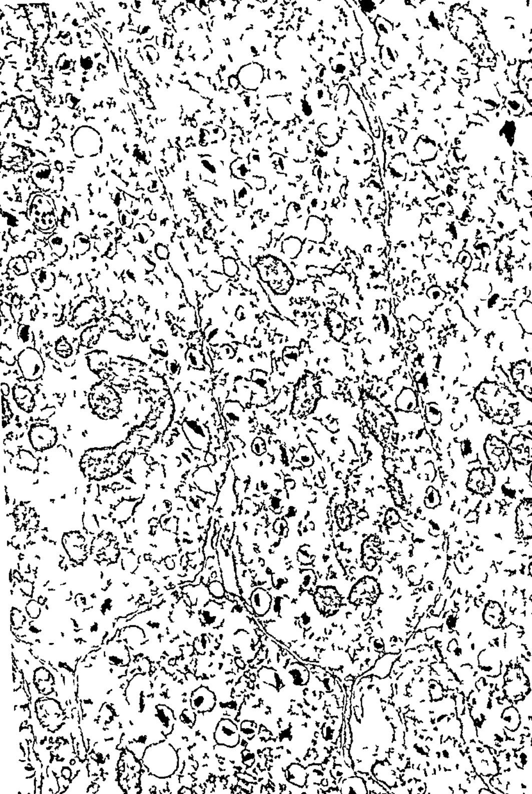


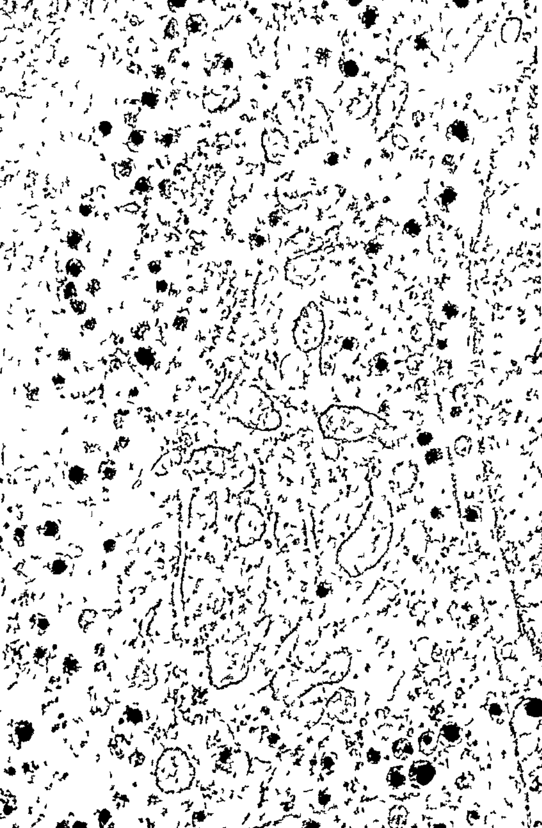
Fig 5 Fluorescence microscopic picture of paragangl on from a ne ιborn rabbit *1?0 ×* Intensely fluorescent cells throughout the entire parenchyma



Fig 6 Fluorescence microscopic picture of paraganglion from a t ιo month old rabbit 1?0 × Only few islets of fluorescent cells remain







1965 b, Brundin Hamberger and Norberg 1966) where NA and A in paraganglia of rabbits have been measured without preceding separation of the amines low values for A have been obtained in most but not in all cases These A values could possibly have been due to calculation artefacts which may occur when measuring NA and A in extracts with large differences between the concentrations of the two amines However the present results indicate that NA is the only CA of reasonable importance for a possible physiologic role of the paraganglia as CA storing organs Therefore the paraganglia have been analyzed only for NA in the quantitative studies

The parallel determinations of CA in both the adrenal glands and the paraganglia of fetal rabbits revealed a marked qualitative difference between the CA contents in the two organs Thus in the adrenal glands A was the predominant CA whereas in the paraganglia NA was the only CA demonstrable This distinct difference seems remarkable in view of the close anatomical relationships between the two organs However the capacity of methylating NA to A in the adrenal glands is not necessarily an inherent property of the adrenomedullary cells An influence of extramedullary factors upon the hormone content of the adrenal medulla has been suggested earlier by several authors (Shepherd and West 1951 Hokfelt 1951 and others) Recently it was reported that hypophysectomy in rats caused a reduction of the adrenal content of the NA methylating enzyme phenylethanolamine *N* methyl transferase and that this reduction could be prevented by the administration of either ACTH or glucocorticoid hormone (Wurtman and Axelrod 1965) Moreover in the paraganglionic tissue of rats where normally NA is the only CA present A has been demonstrated after pretreatment with corticosteroids (Eränkö Lempinen and Kaisanen 1966) — Thus the presence of corticosteroids seems to be required for the formation of A in the chromaffin cell In the adrenal medulla high concentrations of corticosteroid hormones could easily be supplied via the direct vascular connections which have been found between the cortex and the medulla (for ref see Willier 1955) Since no

Fig 7 Survey picture of paraganglionic cells from a newborn rabbit 15 000 × Numerous osmiophilic granules and mitochondria are scattered in the cytoplasm

Fig 8 Paraganglionic cells from a newborn rabbit 36,000 × Most granules exhibit small electron dense cores of irregular shape and large pale peripheries Certain obliquely cut granules exhibit no electron dense material Free and membrane bound ribosomes frequently occur in the cytoplasm A cross section d cilium with 8 + 1 double filaments is seen in the upper left part

Fig 9 Adrenal cells from a newborn rabbit 36 000 × In the medullary cells the cytoplasm contains numerous osmiophilic granules and mitochondria The granules show relatively large rounded electron dense cores the peripheries being dotted and of a certain opacity Layers of thin cellular sheets separate medullary and cortical cells (upper left part)

such vessels from the adrenocortex have been demonstrated in the paraganglia the absence of A in the paraganglia is presumably a consequence of insufficient direct adrenocortical influence

In the *foetus* near term the paraganglia contained about twice as much CA as the adrenal glands The gradually increased NA content in the paraganglia observed during the first postnatal week might reflect a progressive functional development of the storage and/or the synthesis of NA in the organ The considerably increased NA content observed already on the first postnatal day might be explained by a sudden acceleration of this development at birth On the other hand, if the paraganglia were able to release NA continuously during fetal life and this release was interrupted at birth a sudden increase of the organ content of NA could also occur (*cf* Chapter V)

The small individual variations of the NA content in the paraganglia of one and two day old rabbits indicate that animals of this age ought to be the most suitable for comparative studies on the paraganglia This condition was also exploited in the functional studies (Chapter IV and V)

From the fifth postnatal day the individual values showed wider variations within the groups At four weeks after birth the mean NA content of the paraganglia was considerably decreased even below the level for the fetal organs studied This increased variation and final reduction of the NA contents indicate a functional degeneration of the NA storing capacity of the paraganglia

In the histochemical studies the parenchyma of the paraganglia from newborn rabbits was found to consist entirely of cells with large CA contents The appearance of connective and fatty tissues at the expense of the CA containing cells in the paraganglia of two month old rabbits further corroborated that the organs undergo a postnatal degeneration — The earlier concept that the paraganglia are permanent organs in the rabbit (see Chapter I) was based on the observation that the chromaffin reaction was demonstrable also in the paraganglia of adult animals Even though the organ thus might persist in the adult rabbit the present results evidently show that degenerative changes impair the CA storing properties of the paraganglia soon after birth

In the differential centrifugation studies on homogenates of paraganglia and adrenal glands the main portion of the total organ contents of CA was found in the high speed sediments The proportions between sedimentable and non sedimentable CA in the paraganglia were similar to those in the adrenal glands The rate of spontaneous release of CA from the sedimentable fraction of paraganglionic homogenates was measured *in vitro* in a special study

(Brundin 1966) Also in this respect the paraganglia were similar to the adrenal glands — It seems likely that the sedimenting CA were bound to a granular microsomal fraction in these organs as well as in other CA storing tissues (*cf* Chapter 1) It has been shown, however that supernatants obtained by centrifugation of organ homogenates at 1 000 $\times$ *g* contain both the microsomal and mitochondrial fractions of the cells (for ref see Mathias 1966) Therefore, it could not be excluded that some of the CA in the present high speed sediments were bound to other structures than granules On the other hand, since the electron microscopic studies revealed very large amounts of osmiophilic granules in the cytoplasm of both the paraganglionic and adrenomedullary cells from the newborn rabbits, it is reasonable to assume that the major portion of the CA stores was associated with the granular fraction of the cells

In the electron microscope the paraganglionic granules differed characteristically from those of the adrenal medulla in that their electron dense cores were considerably smaller and of irregular shape As judged from the mitochondria in the same sections no general swelling artefact was responsible for this appearance of the paraganglionic granules The paraganglia as well as the adrenal glands were obtained from the same animals and treated identically Thus the paraganglionic granules were different from the adrenomedullary ones The difference observed in the sections might be due to a different reaction to the preparation procedure — The numerous mitochondria observed in the paraganglionic cells indicate that energy consuming processes occur in the cells The large amounts of ribosomes might be correlated to a high capacity of protein synthesis — Cilia have frequently been observed on cells derived from the neural crest (Palay 1961 Dahl 1963 and others) On the chromaffin cells of the adrenal medulla of adult rabbits cilia have been demonstrated (De Robertis and Sabatini 1960) In the present study cilia were observed on paraganglionic cells This might be a sign of the neural origin of the organ

It is concluded that the preaortal paraganglia are large sources of NA in fetal and newborn rabbits and that the organs undergo a postnatal degeneration soon after birth In the newborn rabbit the storage of CA in the paraganglia resembles that in the adrenal medulla in many respects Thus the bulk of the CA stores in the paraganglia is bound to subcellular structures and large amounts of osmiophilic granules are demonstrable in the cytoplasm of paraganglionic cells Even though the appearance of these granules in the electron micrographs is characteristically different from that of adrenomedullary granules many features of the general ultrastructure are similar in the two organs

STUDIES ON THE FORMATION OF CATECHOL AMINES IN THE PARAGANGLIA

In order to study the synthesis of NA in the paraganglionic tissue the incorporation of radioactive CA was measured in the paraganglia from newborn rabbits pretreated with C^{14} labeled CA precursors The adrenal glands from the same animals were used as reference organs The uptake of injected radioactive NA in the paraganglia was also studied

Special methods

Incorporation of radioactive catecholamines from C^{14} labeled precursors

Each of 6 newborn rabbits from the same litter received *dl* DOPA 2 C^{14}* (2 62 μC/μmole) in a single s c injection of 0 12 μC/g b w (total dose of DOPA 9 μg/g b w) The volumes injected were about 1 ml At different intervals after the injections (10 30 and 60 min 24 and 36 hrs) the animals were decapitated and their paraganglia and adrenal glands were excised and extracted The CA of the extracts were then separated by column chromatography the paraganglionic extracts on 5 cm the adrenal extracts on 15 cm Amberlite columns Unlabeled DA was added to the extracts before the separations in order to localize this amine in case radioactivity should appear in these fractions The direct fluorescence and the radioactivity were measured in each fraction of the chromatograms (for details see General methods)

To another litter of 6 newborn rabbits *l* tyrosine C^{14}* uniformly labeled (351 μC/μmole) was given in single s c injections of 0 3 μC/g b w (total dose of tyrosine 0 15 μg/g b w) The paraganglia and adrenal glands of these animals were excised at 1 4 12 24 48 and 72 hrs after the injection of tyrosine C The separation and analysis of CA were performed as described above

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* The radioactive chemicals were obtained from New England Nuclear Corp

Uptake of H^3 labeled noradrenaline

To one newborn rabbit NA 7 H^{3*} (8 1 mC/μmole) was injected in a dose of 0 15 μC/g b w (total dose of NA 4 ng/g b w) At 35 min after the injection the animal was killed and the paraganglion, the adrenal glands and the heart were excised The organs were extracted with 2 ml 0 4 M perchloric acid After centrifugation 500 μl of each extract was measured for radioactivity

Results

Incorporation of C^{14} labeled catecholamines after pretreatment with dihydroxyphenylalanine C^{14}

The incorporation of radioactive CA in the paraganglia and adrenal glands is presented in Fig 10 The total radioactivity values of each CA peak are listed in Table V

Only small amounts of radioactivity were found in the organs excised 10 min after the injection of DOPA C^{14} — At 30 min after the pretreatment, peaks of radioactive NA were found in both the paraganglionic and the adrenal chromatograms In the paraganglia the radioactivity of the NA fractions gradually increased during the whole period studied

In the adrenal glands radioactive NA was rapidly incorporated The maximum peak was obtained 4 hrs after the pretreatment After 24 and 36 hrs only small amounts of NA C^{14} were present in the adrenal glands — Radioactive A appeared in the adrenal chromatograms but it was not until 4 hrs after the DOPA C^{14} injection that a proper peak of A C^{14} was found However at 24 and 36 hrs after the injection most of the adrenal radioactivity was confined to the A fractions

Small amounts of radioactivity were sometimes observed in the DA fractions of the organs removed within one hour after the injection of DOPA C^{14} but no proper peak of DA C^{14} was found in any of the chromatograms

The molar quantities of radioactive CA in the organs were calculated from the values in Table V A summary of the results (Fig 11) shows that the incorporation as well as the spontaneous disappearance of NA C^{14} occurred at a considerably lower rate in the paraganglia than in the adrenal glands

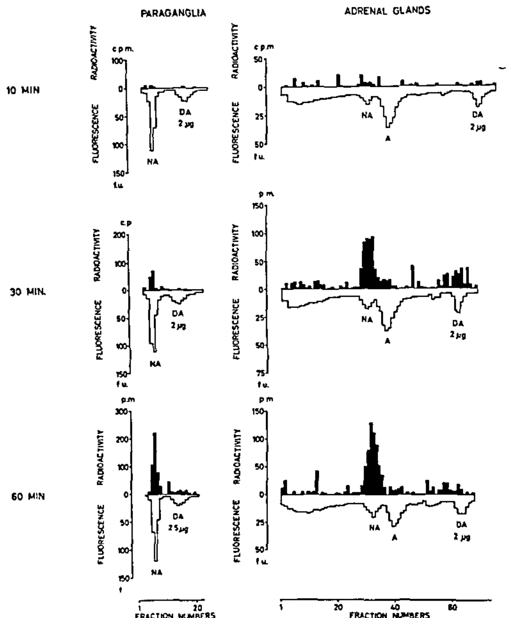
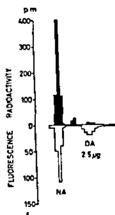


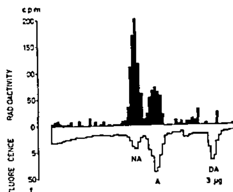
Fig. 10 Chromatograms showing the incorporation of radioactivity in extracts of paraganglia and adrenal glands of newborn rabbits at different intervals after the administration of DOPA-C. DA was added to all the extracts. Separation of CA on 5 cm columns for paraganglion extracts on 1 cm columns for adrenal extracts

PARAGANGLIA

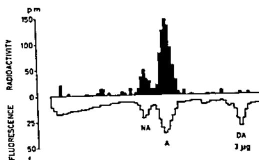
ADRENAL GLANDS

HOURS

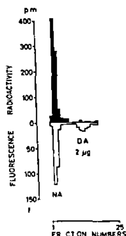


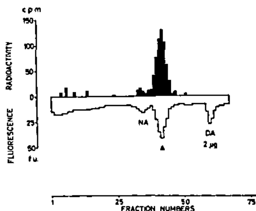


4 HOURS



36 HOURS





FR CT ON NUMBERS

FRACTION NUMBERS

Table I Radioactivity (cpm) within the CA containing peaks from the chromatograms of paraganglia and adrenal glands at different intervals after injection of DOPA C^{14}

Intervals after DOPA C^{14} inj	PARAGANGLIA	ADRENAL GLANDS	
	NA	NA	A
10 min	<100	(119)*	<100
30 »	125	123	<100
60 »	444	[illegible]70	<100
4 hrs	630	690	388
24 »	—	171	696
36 »	[illegible]66	<100	5[illegible]1

* This radioactivity lacked the characteristic peak formed distribution in the chromatogram

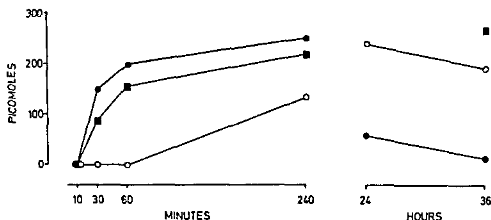


Fig 11 Total amounts of C^{14} labeled CA recovered from paraganglia and adrenal glands of new born rabbits at different intervals after administration of DOPA 2 C^{14} 0 1[illegible] μC/g b w

■—■ NA C in paraganglia
●—● NA C in adrenal glands
O—O A C^{14} in adrenal glands

Incorporation of C^{14} labeled catecholamines after pretreatment with tyrosine C^{14}

The incorporation of radioactive CA in the paraganglia and adrenal glands is presented in Fig. 12 and in Table VI.

In the paraganglion excised one hour after the injection of tyrosine C^{14} only small amounts of radioactivity were found in the NA fractions. However, at all the subsequent intervals studied well defined peaks of radioactivity were observed in the NA fractions of the paraganglia. The maximum amount of radioactive NA was obtained at 24 hrs after the pretreatment and at 48 and 72 hrs the radioactive peaks were reduced in the paraganglionic chromatograms.

In the adrenal glands a clearcut peak of radioactive NA was found already one hour after the pretreatment. At this early stage the adrenal chromatogram was to some extent contaminated by unspecific radioactivity and no proper peak of A C^{14} was observed. At 4 hrs after the tyrosine C^{14} injection a peak of radioactive A had appeared. At the subsequent intervals the amounts of NA C^{14} decreased in the adrenal glands and the main contents of radioactivity were confined to the A fractions. After 72 hrs the adrenal chromatogram was not free from contamination and the peaks of labeled CA were relatively small.

DA C^{14} was not measured in the adrenal glands excised one hour after the pretreatment. In the other chromatograms no radioactivity was found in the DA fractions.

The molar quantities of radioactive CA were calculated from the values in Table VI. A summary of the results (Fig. 13) shows that the maximum amounts of labeled CA were higher in the paraganglia than in the adrenal glands. However, the paraganglia required more time for the accumulation of significant amounts of NA C^{14} and the spontaneous disappearance of radioactive CA was slower from the paraganglia than from the adrenal glands.

Uptake of noradrenaline

The uptake of NA in the paraganglia was evaluated by measuring the radioactivity in the organ at 35 min after an injection of H^3 labeled NA. The radioactivity was also measured in the adrenal glands and the heart for comparison. The amounts of radioactivity in the paraganglion (338 cpm) and the adrenal glands (208 cpm) were very low as compared to that in the heart (7096 cpm).

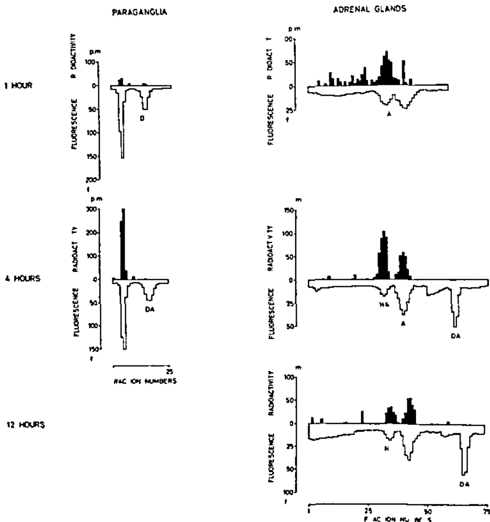
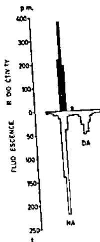


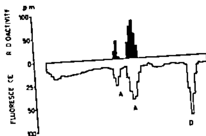
Fig. 12 Chromatograms showing the incorporation of radioactivity in extracts of paraganglia and adrenal glands of newborn rabbits at different intervals after the administration of tyrosine C^{14}. DA was added to all extracts except the first adrenal. Separation of CA on 5 cm column for paraganglionic extracts, on 15 cm columns for adrenal extracts

ADRENAL GLANDS

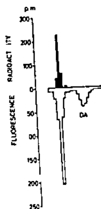
PARAGANGLIA

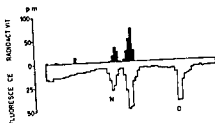
URS



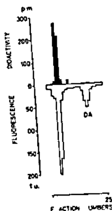


HOURS





72 HOURS



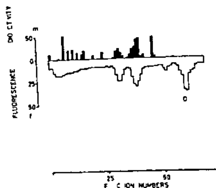


Table 11 Radioactivity (cpm) within the CA containing peaks from the chromatograms of paraganglia and adrenal glands at different intervals after injection of tyrosine C^{14}

	PARAGANGLIA	ADRENAL GLANDS	
Hours after tyrosine C^{14} inj	NA	NA	A
1	< 100	302	< 100
4	603	375	2[illegible]0
12	–	115	2[illegible]5
24	79[illegible]	< 100	325
48	3[illegible]3	< 100	170
72	3[illegible]1	< 100	15[illegible]

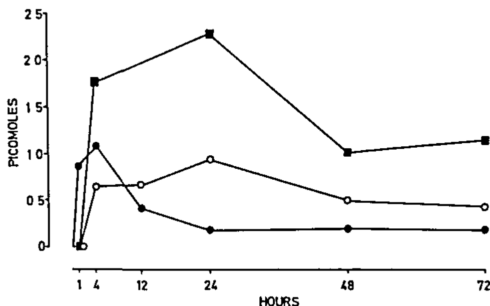


Fig 13 Total amounts of C^{14} labeled CA recovered from paraganglia and adrenal glands of newborn rabbits at different intervals after administration of tyrosine C^{14} (uniformly labeled) 0.3 μC/g b.w.

■—■ NA C^{14} in paraganglia
●—● NA C^{14} in adrenal gland
O—O A C^{14} in adrenal glands

Discussion and conclusions

In two experimental series the incorporation of radioactive NA in the paraganglia was studied *in vivo* at different intervals after the administration of C^{14} labeled CA precursors For comparison the adrenal glands were studied as well — Principally the same technique of CA separation was used for the different organs but the adrenal extracts required a modification of the method for the separation of NA from A The influence of this modification upon the recovery for CA was small in the actual chromatograms as judged from the fluorescence peaks and did not prevent comparative studies of the two organs — Due to the different specific activities of the two precursors used the incorporation of radioactive CA was studied at considerably different quantitative levels in the two series of experiments

The fact that the uptake of injected radioactive NA was very low in both the paraganglia and the adrenal glands indicates that the radioactive CA found in the organs after the administration of the precursors had been synthesized within the organs

The question whether the paraganglionic tissue, like the adrenal medulla is able to perform all the intermediate conversions in the synthetic pathway from tyrosine requires *in vitro* experiments for elucidation In the present *in vivo* study the incorporation of radioactivity was studied in only one of the intermediate products namely DA — In adult mice C^{14} labeled DA has been found in the adrenal glands after the administration of DOPA C^{14} (Rosell Sedvall and Ullberg 1963) and it was suggested that the precursor partly was taken up by the adrenal medulla in the decarboxylated form — In the present experiments no peaks of radioactive DA were obtained at any of the time intervals studied neither in the paraganglionic nor in the adrenal chromatograms Even though this failure to demonstrate DA C^{14} partly could have been due to the relatively low recovery for this amine (see General methods) it might indicate that the conversion of DA to NA in these organs occurred too rapidly to permit an accumulation of DA

After the administration of either DOPA C^{14} or tyrosine C^{14} the maximum amounts of radioactive NA recovered from the paraganglia exceeded those recovered from the adrenal glands However the paraganglionic NA peaks never reached the high specific activity found in the corresponding adrenal peaks (*cf* the low total NA content in the adrenal glands) This could indicate that the capacity of NA synthesis was lower in the paraganglia than in the adrenal medulla — The fact that in both the experimental series NA C^{14} appeared later in the paraganglia than in the adrenal glands strongly suggests that the rate of NA formation is lower in the paraganglia than in the adrenal medulla

The relatively slow spontaneous disappearance of NA C^{14} from the paraganglia might further support this suggestion. On the other hand a direct quantitative comparison of the rates of the spontaneous disappearance of NA C^{14} from the different organs may be misleading, since it can not be excluded that some of the adrenal NA C^{14} represented an intermediate step in the A synthesis and thus disappeared due to methylation. However, in the long term experiment at 72 hrs after the injection of tyrosine C^{14} the amounts of NA C^{14} remaining in the paraganglia exceeded even the sum of the C^{14} labeled NA and A in the adrenal glands. Thus in this experiment the replacement of the radioactive CA was slower in the paraganglia than in the adrenal glands suggesting a relatively lower turnover rate in the paraganglia.

It is concluded that the paraganglia are able to synthesize NA *in vivo*. In spite of the comparatively low rate of the NA synthesis in the paraganglia the relatively large quantities of NA formed by the organs are probably of importance for the physiology of the newborn animal.

CHAPTER V

EFFECTS OF CELLULAR GLUCOPENIA AND ASPHYXIA UPON THE CATECHOLAMINE CONTENTS IN PARAGANGLIA AND ADRENAL GLANDS

It has been shown in studies on adult animals that the dramatic release of CA from the adrenal glands after insulin induced hypoglycemia leads to a reduction of the total adrenal content of CA (Burn Hutcheon and Parker 1950 Hokfelt 1951 and others) Similar effects on the adrenal glands have been demonstrated in animals subjected to cellular glucopenia by the administration of 2 deoxy glucose (2 DG) (Hokfelt and Bygdeman 1961) — In fetal and newborn animals the adrenal glands have been found to release large amounts of CA during asphyxia (for ref see Chapter I page 10)

A study was undertaken in order to find out whether the paraganglionic CA content was affected by experimentally induced cellular glucopenia or asphyxia For comparison the adrenal glands were studied simultaneously

Special methods

Cellular glucopenia

Two litters of 6 newborn rabbits each were used To 3 animals of each litter a 5 5 % aqueous solution of 2 DG was injected s c in two doses of 0 5 mg/g b w each and with an interval of 5 hrs between the injections The remaining animals served as controls and received corresponding volumes of saline s c During the experiments the animals were kept apart from their mothers and were not fed with milk or water At 5 hrs after the last injections the animals were sacrificed The paraganglia and adrenal glands were excised and their CA contents were determined fluorimetrically

Cellular glucopenia was similarly induced in 3 specimens from a litter of 6 two day old rabbits which were all pretreated with DOPA 2 C^{14}, 0 12 $\mu C/g$ b w 33 hrs before The remaining 3 animals were used as controls At 5 hrs after the last injections of 2 DG the animals were decapitated Their paragang

hormone contents of NA isolated chromatographically were measured for radioactivity (for details see General methods)

Asphyxia

Three litters of 2, 4 and 6 newborn rabbits respectively were used. Each litter was divided in an experimental and a control group. The 6 experimental animals were subjected to asphyxia by enclosure in stoppered plexi glass tubes of 100 ml volume. The tightness of the tubes was tested by submersion in water. After 10 min in the tubes the animals were let out and allowed to recover for 10 min. After an additional 10 min period in the tubes the animals were decapitated and their paraganglia and adrenal glands were excised and analyzed for CA.

Hypoxia

Two litters of 6 newborn rabbits each were used. Three animals of each litter served as controls. The other animals were subjected to hypoxia by enclosure for 40 min in plexi glass tubes filled with a gas mixture of 5 % O_2 in N_2. Every second min the gas mixture was blown through the tubes for 30 sec. After a 10 min interval of recovery the procedure was repeated for another 40 min period after which both the experimental and control animals were killed. Their paraganglia were removed and analyzed for CA.

Hypercapnia

In a corresponding material hypercapnia was induced by a similar technique but the gas mixture used was 15 % CO_2 in O_2.

Statistical methods*

The results were treated according to ordinary methods for analysis of variance (for ref. see Snedecor 1956).

* For assistance with the statistical calculations I am greatly indebted to B. Lindström at the Department of Medical Physics, Karolinska Institutet.

Effects of cellular glucopenia upon the catecholamines in paraganglia and adrenal glands

The effects of 2 DG administration upon the total contents of CA in the adrenal glands and paraganglia are presented in Table VII In the adrenal glands the CA content decreased significantly after cellular glucopenia The proportions of NA in the organs varied from 20 to 33 % and were not changed by the treatment In the paraganglia the NA content was not significantly changed but showed a slight increase in the 2 DG treated animals

In order to find out whether the paraganglionic synthesis of NA was affected by cellular glucopenia 2 DG was given to 3 of 6 animals in the paraganglia of which C^{14} labeled NA was previously incorporated by pretreatment with DOPA C^{14} The total amounts of NA C^{14} in the paraganglia were not changed by cellular glucopenia (Table VIII)

Table VII Effects of 2 DG induced cellular glucopenia upon the CA contents of adrenal glands (µg/pair of organs) and paraganglia (µg/organ) in 2 litters of newborn rabbits

		ADRENAL GLANDS				PARAGANGLIA	
	Litter	Glucopenia		Controls		Glucopenia	Controls
		NA+A	% NA	NA+A	% NA	NA	NA
		3 91	27	6 44	25	8 67	7 23
	a	4 43	25	5 03	21	6 90	5 79
		3 57	26	6 75	26	9 21	6 03
Means		3 97	26	6 07	24	8 26	6 35
		3 59	29	5 48	33	7 97	6 27
	b	4 10	29	6 01	20	6 34	6 04
		4 27	20	5 99	28	9 03	8 26
Means		3 99	26	5 83	27	7 75	6 86

Difference
Controls—Glucopenia
P<0 001

Difference
Glucopenia—Controls
P>0 1

hone contents of NA isolated chromatographically were measured for radioactivity (for details see General methods)

Asphyxia

Three litters of 2 4 and 6 newborn rabbits respectively were used Each litter was divided in an experimental and a control group The 6 experimental animal were subjected to asphyxia by enclosure in stoppered plexi glass tubes of 400 ml volume The tightness of the tubes was tested by submersion in water After 10 min in the tubes the animals were let out and allowed to recover for 10 min After an additional 10 min period in the tubes the animals were decapitated and their paraganglia and adrenal glands were excised and analyzed for CA

Hypoxia

Two litters of 6 newborn rabbits each were used Three animal of each litter served as controls The other animals were subjected to hypoxia by enclosure for 40 min in plexi glass tubes filled with a gas mixture of 5 % O in N Every second min the gas mixture was blown through the tubes for 30 sec After a 10 min interval of recovery the procedure was repeated for another 10 min period after which both the experimental and control animals were killed Their paraganglia were removed and analyzed for CA

Hypercapnia

In a corresponding material hypercapnia was induced by a similar technique but the gas mixture used was 15 % CO in O

Statistical methods*

The results were treated according to ordinary methods for analysis of variance (for ref see Snedecor 1956)

* For assistance with the statistical calculations I am greatly indebted to B Lindstrom at the Department of Medical Physics Karolinska Institutet

Table X Effects of hypoxia and hypercapnia upon the total contents of NA (μg) in the paraganglia of newborn rabbits

Litter	Hypoxia	Controls	Litter	Hypercapnia	Controls
	4 90	8 15		4 10	8 14
a	6 45	7 90	c	3 53	7 35
	6 20	7 32		1 50	7 08
	7 42	6 40		5 35	6 35
b	6 03	5 85	d	5 82	5 90
	5 70	7 70		6 80	6 20

Difference
Controls—Hypoxia
P>0 1

Difference
Controls—Hypercapnia
P>0 1

reduced in those animals subjected to asphyxia In the adrenal glands of the same animals the CA contents were not significantly changed Nor did the treatment affect the proportions of the adrenal NA content

In order to perform a more detailed study on the effects of asphyxia upon the paraganglionic NA content two additional experimental series were undertaken in which the NA content was measured in the paraganglia of newborn rabbits subjected to either hypoxia or hypercapnia The paraganglionic contents of NA after these treatments are listed in Table X In two litters the values decreased considerably after hypoxia and hypercapnia alone but in two other litters no overt changes occurred

Discussion and conclusions

It is well known from the literature that certain patho physiological conditions such as hypoglycemia and asphyxia stimulate the release of CA from the adrenal medulla (Cannon and Hoskins 1911 Burn Hutcheon and Parker 1950 Hokfelt 1951 and others) — The effects of hypoglycemia (and of cellular glucopenia following the administration of 2 DG) upon the adrenal CA release are mediated via the splanchnic nerves and can be abolished by denervation of the adrenal glands (Hokfelt and McLean 1950 Hokfelt and Bygdeman 1961) Asphyxia on the other hand has been found to increase the release of CA also from the denervated adrenal medulla This effect of asphyxia seems to be most easily demonstrable in fetal and newborn animals Thus in lambs and calves

subjected to asphyxia Comline and Silver (1958, 1961) found considerably augmented concentrations of CA in the blood drawn from a pouch of *vena cava* at the level of the adrenal veins. In these experiments the NA content of the blood was much more increased by asphyxia than was the A content. Although this result was interpreted as a sign of increased adrenomedullary activity, it was recently suggested that a secretion of NA from the paraganglionic tissue might have reinforced the effect obtained (Comline and Silver 1966). However, no evidence was presented for the existence of such a secretory response from the paraganglia.

An accurate estimation of the release of CA from the adrenal glands (or from the paraganglia) would require analysis of the CA contents in the venous blood from the organs. Since it is technically impossible to catheterize the paraganglionic or the adrenal veins of the newborn rabbit, it is not feasible to perform a proper quantitative study of the CA release from these organs. — However, it is known from studies on adult animals that the release of large amounts of CA from the adrenal medulla is regularly accompanied by a depletion of the CA stores in the organ. In consequence, a significantly reduced CA content in the adrenal glands indicates that a considerable amount of CA has been released from the organs. — This ought to be valid also for the paraganglia. In view of the relatively slow synthesis of CA in these organs a release of significant amounts of CA should readily be reflected by a deficit in the paraganglionic CA stores. — In the present experiments, the release of CA from the paraganglia and adrenal glands was studied in newborn rabbits by measuring the effects of cellular glucopenia and of asphyxia upon the total organ contents of CA.

The fact that the adrenal CA content decreased significantly after cellular glucopenia indicates that the nervous supply to the adrenal medulla is developed up to a functional state already in the newborn rabbit. In the paraganglia the CA content was not decreased by cellular glucopenia, but showed, on the contrary, a remarkable tendency to increase. The mechanism for this slight increase seems difficult to explain. There was no increased rate of NA formation in the paraganglia of those animals subjected to glucopenia, since the replacement of previously incorporated NA-C^{14} by unlabeled NA was not changed in the paraganglia of these animals. These results evidently show that the paraganglia do not release CA parallel to the adrenal medulla. Moreover, the present results support the statement by Muscholl and Vogt (1964) that the paraganglia lack a functional innervation via the splanchnic nerves. This lack of innervation might partly explain the relatively low rate of NA synthesis in the paraganglia (*cf.* Kroneberg and Schuman 1959).

After asphyxia there was a significant reduction of the NA content in the paraganglia. Thus asphyxia elicited a release of NA from the paraganglionic

tissue The mechanism for this asphyxia induced release from the paraganglia might be similar to that for the release of CA from the denervated adrenal medulla demonstrated in asphyctic lambs and calves by Comline and Silver In the present experiments however the CA contents of the adrenal glands did not change significantly There was a slight decrease of the total adrenal CA content and the ratio of NA/A was essentially unchanged in the organs

Since asphyxia is a rather multiform and unspecific method of stimulation, an attempt was made to separate the effects upon the paraganglionic NA content of the two main components of asphyxia hypoxia and hypercapnia Both after hypoxia and hypercapnia the paraganglionic NA contents decreased considerably in certain litters, but these effects could not be consistently reproduced

Even though the asphyxia induced was rather severe, it can not be excluded that also more moderate changes of pO and pCO could exert a stimulation of the NA release from the paraganglia In fact the comparatively low NA content found in the paraganglia of rabbit *foetus* at full term could reflect a deficit of the NA stores due to such a continuous release from the organs

It is concluded that the paraganglia do not give any secretory response to cellular glucopenia and that they lack a functional innervation Asphyxia stimulates the release of NA from the paraganglia probably by a direct action upon the paraganglionic cells The functional role of this asphyxia induced NA release has not been specially studied but it seems likely that this is an important function of the paraganglia not least in view of the common clinical problem fetal asphyxia

SUMMARY

The present work was undertaken in order to investigate the preaortal paraganglia of newborn rabbits with respect to their storage, synthesis and release of catecholamines. In most experiments the adrenal glands from the same animals were simultaneously studied for comparison.

The catecholamine stores in the paraganglia were found to consist of noradrenaline exclusively. Quantitatively, the paraganglionic catecholamine content far exceeded that of both the adrenal glands during the perinatal period. Histochemical studies showed that in the newborn rabbit the parenchyma of the paraganglion entirely consisted of catecholamine containing cells, whereas in the two month old rabbit the organ was degenerated by fibrosis. This postnatal degeneration was accompanied by a reduced organ content of noradrenaline. — The subcellular distribution of noradrenaline in the paraganglia was studied by means of differential centrifugation of organ homogenates. About 70 % of the total organ content of noradrenaline was bound to sedimentable structures. A similar ratio of sedimentable/non sedimentable catecholamines was found in adrenal homogenates. — Electron microscopical studies revealed that the ultrastructure of the paraganglia in many respects resembled that of the adrenal medulla. Large amounts of cytoplasmic osmiophilic granules were found in both the organs. The appearance in the electron micrographs of the paraganglionic granules differed from that of the adrenomedullary ones.

The synthesis of noradrenaline in vivo was found to be comparatively slow in the paraganglia. After the administration of C^{14} labeled tyrosine or dihydroxyphenylalanine both the incorporation and the subsequent spontaneous disappearance of radioactive noradrenaline occurred at lower rates in the paraganglia than in the adrenal glands.

The release of catecholamines from the paraganglia was measured indirectly by determination of the reduction of the organ content of noradrenaline in animals subjected to experimentally induced stressful situations. Cellular glucopenia following the administration of 2 deoxyglucose reduced the adrenal catecholamine content significantly but did not affect the noradrenaline stores of the paraganglia. Since the effects of cellular glucopenia upon the adrenal medulla are mediated via the splanchnic nerves the present results support the opinion of previous authors that the paraganglia probably lack a functional

innervation — Severe asphyxia elicited a release of noradrenaline from the paraganglia reflected by a significant reduction of the noradrenaline content of the organs. No reproducible significant changes were observed after separately induced hypoxia or hypercapnia.

It is suggested that the paraganglia are of functional importance during the perinatal period especially under the common patho physiological condition fetal asphyxia.

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THE REGULATION OF PROXIMAL TUBULAR REABSORPTION IN THE MAMMALIAN KIDNEY

BY
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THE REGULATION OF PROXIMAL TUBULAR REABSORPTION IN THE MAMMALIAN KIDNEY

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BY

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To

Eigil Bojesen

Previously published papers

A Leyssac P P Dependence of Glomerular Filtration Rate on Proximal Tubular Reabsorption of Salt
Acta physiol scand 1963 *58* 236–242

B Leyssac P P, The In Vivo Effect of Angiotensin on the Proximal Tubular Reabsorption of Salt in Rat Kidneys
Acta physiol scand 1964 *62* 436–448

C Leyssac P P, The Effect of Partial Clamping of the Renal Artery on Pressures in the Proximal and Distal Tubules and Peritubular Capillaries in the Rat Kidney
Acta physiol scand 1964 *62* 449–456

D Leyssac P P Some Characteristics of the Proximal Tubular Wall Related to Reabsorption During Luminal Occlusion Following Interruption of Glomerular Filtration
Acta physiol scand 1965 *63* 36–45

E Leyssac P P, The Luminal Occlusion Time of Proximal Tubules in Young Rats
Acta physiol scand 1965 *64* 176–181

F Leyssac, P P The In Vivo Effect of Angiotensin and Noradrenaline on the Proximal Tubular Reabsorption of Salt in Mammalian Kidneys
Acta physiol scand 1965 *64* 167–175

G Bojesen E and P P Leyssac The Kidney Cortex Slice Technique as a Model for Sodium Transport In Vivo A Qualitative Evaluation
Acta physiol scand 1965 *65* 20–32

H Bojesen E, P P Leyssac and B Svejgaard Nielsen Analysis of the Efflux Kinetics of Sodium from Small Cylinders of Rabbit Kidney Cortex
Acta physiol scand 1965 *65* 105–119

Table of contents

Introduction

When introduced to renal physiology the student is impressed first by the wide aspects and possibilities opened with the development of direct methods in the study on water and solute transfer across the glomerular membrane and across the different segments of the tubular wall along the nephron Since the time of the original micropuncture experiments on frog kidneys 40 years ago the microtechniques and analyses have been widely applied with increasing refinement and perfection to studies on transfer processes in single nephrons of the mammalian kidney especially during the last decade Thus the renal physiologist has gained a rather perfect tool for investigations of the different role played by glomerular and tubular factors in changes observed in the excretion of water and solutes

During recent years the direct methods have enlarged our understanding of the movements (reabsorption and/or secretion) of the individual constituents of the glomerular ultrafiltrate in the different segments of the whole nephron these methods have provided also a certain information concerning the individual processes of the transcellular transfer viz whether they are ascribable to passive or active energy requiring forces

The introduction of these direct methods has contributed greatly also to the establishment of our present theory of the renal mechanisms by which a concentrated and dilute urine in the mammalian kidney is elaborated consequently it has contributed also to our understanding of the osmoregulation of the extracellular fluid space In this model the structural and functional correlations have been synthetized into a system which in a simple and ideal way seems suitable for a hormonal regulation of water excretion independent of simultaneous solute excretion

Conversely, the renal handling of salt seems to be a problem which is becoming increasingly complicated even though innumerable data concerning the integrated renal handling of salt have been obtained by clearance studies on the renal salt excretion in mammals studies which have been carried out under a great variety of experimental conditions normal and pathological states and under the influence of various hormones and other substances Data obtained by direct methods from the different segments of the nephron have failed to support some indirect evidence obtained from clearance studies as will appear from the following analysis, many of the data are seemingly controversial or obscure The link by which the functional states of the

proximal and distal tubular segments are correlated, no doubt a sine qua non for a regulation of the salt and water excretion, has been searched for although without success Further, any synthesis of the accumulated knowledge into a model which is generally acceptable on the basis of our present knowledge and which describes in quantitative terms the renal mechanisms involved in the regulation of the salt excretion, and thereby of the volume of the extracellular space, has not been proposed This fact may appear rather surprising since it had been possible at an earlier stage to describe in quantitative terms the process of transfer of another reabsorption substance, viz glucose and to bring into accordance the directly obtained knowledge concerning the function of the proximal tubular segment and the integrated tubular functions observed in clearance studies

Hence it seems justified to question whether or not some essential feature in the renal handling of salt actually has been recognized as a problem worth investigation by a direct method It is my intention in the present work to draw the attention primarily to one feature in the renal handling of salt which so far and with one single exception has not been fully recognized as a problem of major importance The problem was clearly formulated by the question whether or not the rate of tubular transfer of the major part of filtered salt and water is dependent on the filtered load per se In order to make the reader comprehend the origin of this problem the difficulties involved in the recognition and solution of the problem, and the inferences of a final answer to the question a brief and far from exhaustive review shall be given of the basis of the former generally accepted theory and the previous interpretations hereof

In a subsequent discussion the problem thus raised is analysed on the basis of available data, the greatest importance being attached to data obtained by direct methods Based on the conclusions drawn from this analysis and on basis of further experimental approaches to the problem of the regulatory mechanisms if any of the proximal tubular reabsorption of salt and water I shall attempt to bridge the gap between findings concerning the function of the single tubular segments in transfer of salt and water obtained by direct methods and the indirect evidence of the integrated function obtained from clearance studies Finally it may turn out I hope that it will be possible on this basis to give an outline of some of the major features of the renal mechanisms involved in the regulation of the salt excretion and to correlate these functional concepts to macro and ultrastructural equivalents

It has been endeavoured first and foremost to restrict the present review exclusively to data of essential importance to the problem concerned rather than to give any exhaustive review and interpretation of the avaible data

CHAPTER I

A EARLY CONCEPTS OF THE MECHANISMS IN URINE FORMATION

a) *Glomerular filtration – tubular reabsorption*

In the fundamental micropuncture experiments on frog kidneys Wearn and Richards (1924) directly established the two main principles outlined in Cushny s modern theory (1917), the processes of glomerular ultrafiltration and of tubular reabsorption They demonstrated that fluid collected from the space of Bowman s capsule did not contain protein in measurable concen trations but contained chloride and glucose, while simultaneously collected final urine was almost free of these components in the filtrate

b) *Determination of the glomerular filtration rate*

The requirements to a quantitative characterization of the tubular transfer processes of the separate components of the ultrafiltrate seemed fulfilled by the introduction of a quantitative method by which to estimate the rate of glomerular filtration of water (GFR) in the intact kidney by measurement of exogenous creatinine clearance (Rehberg 1926 a) By this indirect method it became possible to calculate the rate of reabsorption of a filtered substance as the difference between the rate of filtration and the rate of excretion of the substance studied

It is outside the scope of this review to enter into a discussion about the objections later raised as regards the correctness of using the exogenous creatinine clearance as a measure of filtration rate, especially in human beings and apes Only it shall be mentioned that Shannon and Smith (1935) demon strated in normal human subjects that the clearance of inulin is independent of its concentration in plasma and somewhat lower than the creatinine clearance Furthermore Shannon (1935 a) showed that the creatinine clear ance was reduced to values approximately equal to values of inulin when the plasma concentration of creatinine was greatly increased and also during phlorizination In later clearance investigations inulin has become the standard substance of choice for measurements of the glomerular filtration rate

Further insight into partial processes in urine formation was conditioned by the quantitative determinations of the filtration rate single milestones on

the way to the generally accepted theory of the renal mechanisms of urine formation shall be briefly mentioned to form a basis of the following discussion

c) *Passive – active reabsorption*

On the basis of clearance studies on the excretion of urea, Rehberg (1926 b) first emphasized the necessity of a distinction between passive back diffusion and active reabsorption (in the sense of reabsorption against a concentration gradient) He presented evidence of passive diffusion of urea from the filtrate to the plasma showing that the concentration index of urea always was lower than that of creatinine and that it decreased relatively with increasing concentration indices of creatinine and vice versa Later investigations using direct methods have not yet been able to establish any active transfer of urea in the mammalian kidney

Several investigators have found that the excretion fraction of glucose, water, sodium and other substances may vary more or less independently and consequently Cushny s theory of the reabsorption of an ideal fluid of constant composition was completely abandoned, a theory which on the basis of similar arguments had been questioned even by Cushny himself However, because of the low excretion fraction of these substances it goes without saying that the result only permits the conclusion that the theory of a constant composition does not apply to the total reabsorption It was not excluded that some possibly a great fraction of the filtrate is reabsorbed as a perfected Lockes fluid

d) *Tubular secretion*

The participation of tubular secretion in urine formation previously suggested by several investigators on the basis of experiments with certain dyes as indigo carmine and phenol red was clearly demonstrated by Marshall (1930) in aglomerular kidneys of marine teleosts, the goosefish *(Lophius piscatorius)* and the toadfish *(Opsanus tau)* He observed that flow rate and composition did not differ essentially in urine obtained from glomerular as opposed to aglomerular marine teleosts From both types of fish large amounts of magnesium sulphate phosphate creatinine, and undetermined nitrogen, and small amounts of urea were found to be characteristic components of the urine Both types of fish can eliminate phenol red but a specificity in the elimination of certain foreign bodies and carbohydrates was observed Only the glomerular kidneys were capable of eliminating ferrocyanide and glucose

even when the concentration of glucose in plasma was high and phlorizin had been given

In clearance studies a tubular secretion of a substance is indicated only when its clearance is higher than the simultaneous clearance of inulin under steady state conditions In this way tubular secretion of phenol red was demonstrated in dogs by Shannon (1935 b) who furthermore discovered that the clearance of phenol red decreased to values approaching or even below the simultaneous clearance of creatinine provided the plasma con centration of phenol red was increased above a certain level This phenomenon (self depression), which has now been demonstrated for a large number of organic compounds was believed to be due to a saturation of some step in the transfer mechanism suggesting a maximal transfer capacity (Tm or T max)

e) *Tm limitation of rate of transfer of actively reabsorbed substances*

An apparently similar limitation in the rate of tubular reabsorption was demonstrated and clearly delineated in quantitative terms for glucose by Shannon and Fisher (1938) With increasing plasma concentration of glucose the quantity of glucose filtered would increase in direct proportion to the plasma concentration at a constant filtration rate but glucose did not appear in the urine until a certain critical plasma level had been reached the renal plasma threshold Above this plasma level the quantity of glucose excreted per unit of time is also a linear function of the plasma concentration The difference between the amount filtered and excreted per unit of time equal to the quantity reabsorbed per unit of time was found to be constant (maximal) over a great range of plasma concentrations and for prolonged periods of time In other words the rate of reabsorption of glucose above the saturation level is independent of the filtered load (load is defined as (the volume × the concentration) supplied to the cells per unit of time) Shannon considered this finding of a maximal reabsorptive capacity for glucose (Tm_G) due to the fact that each tubule cell is unable to transfer more than a certain (maximum) quantity of the substance per unit of time In the following dis cussion the term Tm is used exclusively in accordance with this original definition which, of course does not exclude that the actual Tm value may be subject to hormonal regulation

The concepts of urine formation thus delineated might be described in brief as a primary glomerular ultrafiltration followed by propulsion of the filtrate along the tubular lumina attributable only to a hydrostatic pressure head determined by the difference between the pressure in the space of

Bowman's capsule and the renal pelvic pressure During the flow of filtrate along the tubular lumina a certain fraction of the filtered water and solutes is reabsorbed, either by passive back diffusion (e g urea and other waste products) or for other substances – the Tm substances – by transfer mechan isms of limited reabsorptive capacity Still other filtered substances may not be reabsorbed at all, while some organic compounds may be excreted in the urine by mechanisms involving in part tubular secretion of limited rates of transfer

B BASIS OF THE ACCEPTED THEORY

a) *Assumption I Clearance of inulin a measure of glomerular factors*

Rehberg s estimation of glomerular filtration rates in clearance studies of 100–150 ml/min indicated that about 98–99 per cent of filtered salt and water is reabsorbed in the normal human kidney, an observation which later has been confirmed in almost any mammal investigated, in the physiological state the excretion of sodium chloride will never exceed 5–10 per cent of the filtered load In accordance with the filtration reabsorption theory and on the basis of simple hydrodynamic considerations such low excretion frac tions of salt and water would indicate that any interference with the tubular reabsorptive capacity instantaneously will reduce the rate of filtration, as realized clearly by Rehberg (1929) He writes Es ist doch ziemlich ein leuchtend dass der nach der Filtration restierende Druch der also dazu aus reichen soll erst die totale Filtratmenge bis zu der eventuellen Resorptions stelle und dann die viel kleinere übrigbleibende Harnmenge zur Blase zu treiben, nicht ausreichen kann um die totale Menge zur Blase zu treiben Eine Lahmung der Ruckresorption muss augenblicklich eine starke Herabsetz ung der Filtration herbeifuhren

It might have been relevant to ask now whether this also applied to such parallel changes in the rate of filtration and reabsorption of salt and water which occur physiologically, i e whether or not the spontaneous range of variations in the filtration rate (about 80–140 ml/min/1 73 m surface area in man), which might occur without significant changes in the rate of excretion of salt and water, is determined by primary changes in the rate of reabsorption But unfortunately this natural question was not asked On the contrary it was generally accepted without critizism or discussion that the determination of the glomerular filtration rate is a pure measure of the functional state of the glomerular vessels and that occurring changes in the rate are brought about only on account of changes in the vascular resistances

in the afferent and/or efferent arterioles Actually the term glomerular filtration rate was apparently a conceptual trap It shall be emphasised that this assumption, which till now has not been experimentally confirmed directly, formed the basis upon which Homer Smith built his theories on the renal handling of salt and water, as they are outlined in his book The Kidney (1951)

The clear logic in Rehbergs above quoted statement may have been neglected by later investigators or taken only as an indication of a gross functional glomerulo tubular balance , which most recently has been subject to a careful experimental investigation by direct methods Potassium di chromate or mercuric chloride was administered to rats in order to provoke necrosis of different parts of the tubular segments After stated periods of time blood sampling, urine collections, and micropunctures of proximal and distal tubules were performed by Gottschalk and his group (Biber Mylle and Gottschalk 1964) Clearances of inulin tubular fluid to plasma ratios (F/P) and urine to plasma ratios (U/P) of osmolality inulin, urea, and para amino hippurate (PAH) were determined The tubular lesions and sites of punctures were analysed by microdissection and described by Oliver and MacDowell (1964) A good structural functional correlation was observed as regards extent and localization of the tubular necrosis and functional disturbance On an average the inulin clearance (51 measurements) was reduced by two thirds in the dicromate animals, in which the tubular necrosis was approximately 60 per cent of the proximal convoluted segment in accordance with Rehberg s prediction Furthermore the F/P ratio of PAH and urea in distal tubules were greatly reduced as compared to normal controls When however these F/P ratios were divided by the F/P ratio of inulin from similarly located puncture sites the ratio was not significantly different from that observed in normal rats Since the F/P ratio of inulin depends primarily upon the proximal reabsorption of sodium and water, and since necrosis of this segment will affect sodium and water reabsorption as well as the ability to secrete PAH a normal ratio (F/P PAH divided by F/P inulin) in injured kidneys indicates merely that the reabsorption capacity and the ability of secretion is equally impaired, in accordance with expectations and the conclusions drawn

b) *Assumption II Proximal sodium reabsorption load dependent Distal sodium reabsorption Tm limited*

A very close parallelism between the filtered load and rate of reabsorption of salt and water which was established in numerous clearance studies in

the years between 1930–1940, suggested furthermore an intimately adjusted *physiological* glomerulo-tubular balance This fact offered serious difficulties of comprehension and quantitative characterization of the transfer process involved according to the current concepts of the Tm limitation of the 'active ly' reabsorbed threshold substances Micropuncture experiments in rats by Walker et al (1941) demonstrated that by far the major fraction of the filtrate was reabsorbed proximally, as suggested by Rehberg (1926 a and b) By analyses on fluid collected from the proximal tubules these authors showed that the F/P ratio of creatinine had increased to a value of about 2,5 (2 3) halfway down the convoluted segment indicating by extrapolation that about 75–80 per cent of the filtered load of water had been reabsorbed at the end of the proximal convoluted segment Furthermore the osmotic pressure of proximal fluid was found to be isosmotic with the plasma regardless of the site of puncture, indicating that a similar fraction of the osmotically dominant solutes (sodium, chloride and bicarbonate) had been reabsorbed at this site These observations have been repeatedly confirmed by later investigators using the same direct method (e g Lassiter, Gottschalk and Mylle 1961 Windhager and Giebisch 1961 b) Altogether the findings indicated that changes in the distal reabsorption of salt and water will have but insignificant effect upon the total reabsorption of either constituent, as long as the fraction of salt and water reabsorbed proximally is large and remains fairly constant The absolute amounts of both salt and water reabsorbed per unit of time will change in almost direct proportion to the filtration rate as long as the rate of excretion of salt is low as compared with the filtered load Indeed the rates of filtration and reabsorption of salt are normally very nearly equal and hence the rate of reabsorption appears to vary in direct proportion to the filtered load as stated by Smith (1951, p 312) The implication in this statement is that the filtered load of salt represents the independent variable parameter in accordance with the prevailing assumption According to this assumption (that the determination of inulin clearance is a measure solely of the functional state of glomerular vessels) it might be possible theoretically to characterize the process of salt and water reabsorption quantitatively by the clearance method In this case apparently the tubular reabsorption rate of salt would be an unanalysed function of the filtered load The concept of a salt Tm was thereby invalidated and could hardly be maintained If on the other hand the rate of filtration were determined by the rate of reabsorption of salt and water, as it might be if the above quoted statement by Rehberg (1929) were to apply also to normal conditions any characterization of the transfer process of salt and water by clearance measurements would of course be meaningless

It was realized however that a distinction between glomerular and tubular

factors using the clearance method involved great difficulties with respect to salt and water, since the variability of the glomerular filtration rate during registration may be appreciable, successive measurements cannot be made in which variations between the individual measurements are less than 5–10 per cent indeed a very large variability as compared with the rate of salt excretion Hence reabsorption rates of salt can only be evaluated under identical conditions of plasma sodium concentrations and filtration rates (i e of filtered loads) and relationships of excretion rate to load and capacity cannot be evaluated during, or immediately following a change in the load using the conventional clearance technique as emphasized in several reviews (e g Smith 1951, Wesson 1957, Kruhoffer 1960)

c) *Assumption III–IV Proximal sodium reabsorption normally gradient limited Urea contributes to establishment of the gradient*

A further and essential step to a seemingly acceptable theory of the mechanisms of renal tubular transfer of salt was presented by Wesson Anslow and Smith (1948) These authors showed in dogs that as much as 60–65 per cent of the filtered water may be excreted in the urine during osmotic diuresis induced by infusion of mannitol solutions while at the same time only 15–25 per cent of the filtered sodium is excreted (indeed a considerably increased excretion fraction of sodium) In these experiments the flow of urine was very great and possible changes in the composition of the urine affected by the distal tubules might safely be considered relatively insignificant for which reason the composition of the final urine might be taken as an expression of the composition of the tubular urine at the end of the thin limb of Henle This observation demonstrated that the reabsorption of sodium in the proximal tubules to some degree might be independent of the reabsorption of water and that sodium reabsorption in this segment is an active process which may proceed against a chemical (concentration) gradient This finding was confirmed in similar experiments in rabbits by Kruhoffer (1950) direct evidence from later micropuncture studies have fully established the active character of the proximal sodium transfer

By the method of stop flow perfusion of single Necturus proximal tubules Solomon and his associtess (Shipp et al 1958) introduced into the lumen perfusion fluids containing different concentrations of sodium chloride and low concentrations of ^{14}C labelled inulin The fluids were made isosmotic to Necturus plasma by addition of mannitol By analysis of the inulin concentration in the fluid collected at the end of the experiment from the lumen blocked by mineral oil on each side of the perfusion fluid the percentage of

water reabsorbed was calculated by the degree to which the inulin had become concentrated They showed that fluid can be reabsorbed from a solution containing sodium chloride in concentrations even down to 65 meq/l into plasma containing 100 meq/l (Windhager et al 1959) With a lower salt concentration in the perfusion fluid water would move into the luminal fluid from the outside These experiments indicate an active reabsorption of sodium or chloride, water following passively, if water were actively transported it might be difficult to understand why it could not be reabsorbed from solutions of low as well as high salt concentrations in which osmotic pressures were identical These observations were confirmed in similar experiments in rats by Windhager and Giebisch (1961 a) in which a limiting concentration gradient of 10-60 meq/l was established within a few seconds Similar findings during free flow conditions of sodium chloride reabsorption against a concentration gradient induced by mannitol infusions in rats were also reported by Windhager and Giebisch (1961 b), altogether indicating that sodium and/or chloride ions are actively transported

The demonstration first by Solomon (1957) and by Giebisch (1958) of a transtubular electrical potential difference in proximal tubules of rat kidneys of 19–39 mV (on an average 20 mV), which was reversibly abolished by interruption of the renal blood circulation, indicated that sodium ions are actively transfered across the cellular wall Measurements by Giebisch (1958) and Whittembury (1960) of an electrical assymetry in the proximal tubular cells of Necturus, in which a potential difference (P D) of 74 mV acro s the peritubular membrane (cell negative) and a transtubular P D of 20 mV (lumen negative) was recorded, indicated a P D of 54 mV (cell negative) across the luminal membrane This observation suggested a localization of the active step in the transfer process at the peritubular side of the cell Finally Eigler (1961) using Necturi and Windhager and Giebisch (1961 a) using rats have demonstrated that short circuit currents obtained from single proximal tubules are approximately equivalent to net sodium reabsorption measured under similar experimental conditions Thus in agreement with the conclusions drawn by Wesson Anslow and Smith (1948), it seems safe to consider the proximal sodium reabsorption as an active process proceeding against a chemical and/or electrical gradient (uphill transport)

The increased excretion fraction of sodium to values well above 20 per cent found in the above cited experiments of osmotic diureses by Wesson Anslow and Smith was as umed to be due to the flow rate (greater load) which had risen to a level above the capacity of the distal tubules The observation in these latter experiments that a concentration gradient might have a rate limiting effect on the sodium reabsorption rate in the proximal tubules was

in striking contrast to the apparent lack of such gradient limitation of distal tubular sodium reabsorption since it was known that almost all sodium and chloride may be removed from the urine under conditions of salt depletion Furthermore, experiments on volume expansion by rapid infusion of Locke s fluid (e g Wesson et al 1950) had shown that the rate of excretion of sodium had rapidly increased (within 20–30 min) parallel with increases in the glomerular filtration rate and renal plasma flow Conversely studies on the effect of changing the renal arterial pressure showed that reduction in one of the kidneys of the local arterial pressure below 80–90 mm Hg was accompanied by reductions both in the rate of filtration and in the rate of excretion of sodium (Selkurt, Hall and Spencer 1949) This result was confirmed in subsequent publications by Blake et al (1950), Mueller et al (1951) and Selkurt (1951 a), who also observed that minor reductions in the renal arterial pressure on one side might reduce the sodium excretion rate on this side without affecting the rate of filtration to any detectable degree These latter observations which shall be further discussed in a later chapter were apparently not considered to be significant and did not throw any serious doubt on the idea of a load-dependent sodium reabsorption

In order to correlate the above and other observations into a satisfactory system the hypothesis was first proposed by Wesson Anslow and Smith (1948) and later elaborated by Smith (1951) that the reabsorption of sodium takes place in two stages (1) a *proximal process* involving approximately four fifths of the normal filtered load which process is a linear function of the load (2) a *distal process* involving approximately one fifth of the filtered load which in contrast to the proximal tubular process is rate limited by a maximal transfer capacity (Tm^{d}_{Na})

The evaluation of the experiments underlying these hypothetical concepts are evidently open to critizism and severe sceptisism since the clearance technique used in these investigations by no means was designed to follow a rapid sequence of events Consequently the results obtained could not establish whether the observed changes in the rate of filtration were caused by possible primary changes in the rate of proximal reabsorption (changes of a capacity) or whether such changes in the transfer rate were events – automatically adjusted – secondary to changes in the filtered load The same argument applies also to the usual interpretations of recent micropuncture data which are said to support the theories advocated by Smith It was shown that the same fraction (75–80 per cent) of the filtered volume had been reabsorbed at the early part of the distal tubules under antidiuretic conditions (Lassiter Gottschalk and Mylle 1961) as well as under conditions of isotonic or hypertonic salt loading (Giebisch Klose and Windhager 1964) in spite of consider

able increases in the glomerular filtration rate under hypertonic loadings. Similarly insignificant differences only were observed in this fraction of the filtered volume reabsorbed proximally in antidiuresis and diabetes insipidus, in spite of an about 50 per cent reduction in the filtration rate in the latter condition (Gertz, Kennedy and Ullrich 1964). Again these investigations do by no means permit the distinction between primary and secondary events, since the collections of luminal fluid were not completed nor initiated until several minutes (more than 15 min) after the load had changed. Therefore the results do not at all prove, nor do they even permit the suggestion, that the proximal reabsorption rate has changed as an automatical consequence of the load eo ipso.

In order to explain such postulated automatical adjustment of the proximal rate of sodium transfer, Smith and his collaborators inferred on the basis of the observation of a proximal rate limiting concentration gradient for sodium during osmotic diuresis that the reabsorption af sodium also *normally* occurs against a moderate concentration gradient (see Smith 1951, p. 311). It was further assumed that only a small fraction of filtered urea is reabsorbed proximally. Thereby the osmotic effect of urea might supply a basis for the establishment of the concentration gradient for sodium in addition to a relatively retarded diffusion of water. Thus increases in the filtered load would serve to diminish (or eliminate) the concentration differences by increasing the volume of fluid presented to the tubules. Thereby the absolute rate of sodium reabsorption would tend to increase pari passu with the filtration rate and vice versa. But naturally, evidence obtained under conditions of mannitol diuresis does not allow any such inference to mechanisms operating under physiological conditions. Furthermore a few direct observations in rats on the sodium concentration in the proximal tubular fluid and in plasma (Walker et al. 1941) during moderate saline diuresis had already indicated that no such gradient was established – at least not half way down this segment. These direct observations have been confirmed in several later micropuncture studies in rats during saline diuresis and antidiuresis (e.g. Windhager and Giebisch 1961 b, Ullrich et al. 1963). It was shown unequivocally that the fluid to plasma ratio of sodium (F/P Na) in the entire convoluted part of the proximal tubules remains as close to unity, as might be obtained with the analytical techniques applied. Also it was shown (Lassiter, Gottschalk and Mylle 1961) that about 50 per cent of filtered urea is reabsorbed at the end of the proximal convoluted segment (about 60 per cent of the total proximal length). In this context a more essential importance is attached to their demonstration that the proximal F/P urea, which increases in the most early part of this segment (first 10–15

per cent) to an average of 1 5 shows no tendency to increase or to decrease further along the accessible part of the convolution, indicating that urea is reabsorbed along with sodium and water in the same proportion as in the tubular fluid Hence the osmotic activity of urea cannot contribute to the establishment of a concentration gradient for sodium in this segment

d) *Assumption V The constancy of the hydrostatic pressure in the space of Bowman s capsule*

A highly fundamental consequence or underlying assumption implied in the generally accepted concept of a load dependent proximal sodium reabsorption is the postulate that the hydrostatic pressure in the space of Bowman s capsule were a constant parameter equal to the interstitial pressure notwithstanding the great changes in the rate of filtration The flow of fluid across the glomerular membrane and further along the tubular lumina until the renal pelvis must be brought about by a common pressure head by Kruhoffer (1960) termed the glomerular propulsive pressure which is equal to the difference between (the hydrostatic pressure in the glomerular capillaries – the colloid osmotic pressure of the plasma proteins) and the renal pelvic pressure A part of this pressure head the effective filtration pressure (viz glomerular propulsive pressure – hydrostatic pressure in the space of Bowman s capsule) is used for the formation of the total filtered fluid volume Apparently the general assumption that changes in the glomerular filtration rate are caused primarily and exclusively by glomerular factors (i e by changes in the glomerular propulsive pressure) implies that the capsular pressure must be constant

Within the accuracy of the available technique any significant pressure decline along the proximal convoluted segment cannot be demonstrated (Gottschalk and Mylle 1956) and consequently the resistance to fluid flow along this segment must be very low Under antidiuretic conditions the control pressure averaged in the proximal tubules 12 5 mm Hg and in the distal tubules 6 7 mm Hg (Gottschalk and Mylle 1957), indicating a considerable resistance across the loop of Henle During osmotic diuresis the distal intratubular pressure increased to values almost identical to those measured in the proximal tubules under these conditions, indicating a further considerable resistance to flow at a more distal site, which might be rate limiting to flow at high urine flow rates The ducts of Bellini were considered to be the site of this distally located resistance Since the major fraction (75–80 %) of the filtered volume is reabsorbed proximally, it seems evident that an increase in the glomerular propulsive pressure (accelerating the filtration rate although

not necessarily to measurable degrees) will be followed by an almost parallel increase in the proximal intratubular (viz capsular) pressure – i e a changed balance between tubular resistance and volume of fluid presented – unless either the resistance is reduced or the rate of proximal reabsorption is increased According to the opinion of Smith such increase in the reabsorption rate would be an automatic secondary event as outlined above If, on the other hand, the rate of proximal reabsorption were independent of the filtered load (i e a process of Tm character above the saturation level) any increase in the glomerular propulsive pressure would be followed by a similar change in the capsular pressure and no significant (measurable) change would occur in the effective filtration pressure (or glomerular filtration rate) until some other factor(s) change(s) the reabsorptive capacity (or tubular resistance)

Indirect evidence against the implication of a constancy in the capsular pressure following changes in the glomerular propulsive pressure was presented by Bojesen (1954 b) In order to measure correctly inulin clearance in dogs even under conditions of changing urine flow, corrections were applied to avoid errors originating in tubular delay (at low urine flow) and dead space errors by determinations of appearance time' of injected dye and determinations of renal pelvic volumes at different urine flow rates (Bojesen 1954 a) By this careful procedure the sequence of events in dogs was studied within the first 10–15 min following rapid infusions (200–300 ml per 20 kg body weight) either of colloid free Ringer solutions (dilution diuresis) or Ringer solutions containing high concentrations of dog plasma proteins It was shown that changes in the oncotic pressure of the plasma (and thereby in the glomerular propulsive pressure) by 8 mm Hg failed to change significantly ($\geq$10 %) the rate of glomerular filtration within the first 10 min Renal plasma flow (clearance of diodrast) remained also unaffected within this period of time After 10–15 min the glomerular filtration rate and the renal plasma flow had increased to measurable degrees On the assumption of constancy in the capsular hydrostatic pressure a change by 8 mm Hg in the glomerular propulsive pressure should change significantly the rate of filtration unless the pressure drop across the glomerular membrane were to exceed about 80 mm Hg which is incredible, or unless the resistance in the afferent and/or efferent arterioles had changed The latter possibility seemed unlikely since the renal plasma flow remained unaffected Therefore it was justified to conclude that because of a parallel change occurring in the capsular pressure the effective filtration pressure had not changed significantly within the first 10 min This indirect evidence which is incompatible with the theories advocated by Smith, is confirmed by direct measurements of the

proximal intratubular pressure in rats under similar conditions (Gottschalk and Mylle 1956) These authors injected relatively similar amounts of dextrose solution (1–2 ml per 200–300 g of body weight) intravenously and observed by rapidly successive recordings of the intratubular hydrostatic pressure, an immediate pressure increase to an average of 11 mm Hg (5–25 mm Hg) The pressure then declined gradually to preinjection values in 4–19 min averagely in 7 5 min It is apparent from the figure that the mean systemic arterial pressure had not changed significantly at the peak of intratubular pressure increase (Gottschalk and Mylle 1956, fig 2) and the authors found no correlation between arterial and intratubular pressure Although not discussed in their paper these direct observations agree remarkably well with Bojesen s results obtained by the modified clearance technique with respect to the absolute value of the pressure increase as well as the time interval between the lowering of the oncotic pressure and until significant changes in effective filtration pressure occurred

Although it is not my intention here to enter into a profound discussion and provide an interpretation of the many phenomena linked to the concept of autoregulation of renal blood flow and filtration two different types of response to the elevation of the renal arterial perfusion pressure may be of relevance in the present discussion since they may illustrate the significance of the intratubular pressure change to the effective filtration pressure, as indicated by the above observations The phenomenon of an absence of changes or of moderate changes only in the renal blood flow and the glomerular filtration rate at changing renal perfusion pressures within the interval of 80 to 180 mm Hg has been extensively investigated under a variety of experimental conditions and by different techniques Changes in the renal tissue pressure originally measured by the now almost abandoned needle technique , are measured by the wedged deep intrarenal venous pressure or by micropuncture of the proximal tubules and peritubular capillaries These two methods give almost identical results as demonstrated by Gottschalk (1964 a) It has been observed repeatedly especially by Hinshaw and his colleagues (see Hinshaw 1964) that the renal tissue pressure changes parallel with arterial perfusion pressure within the interval of autoregulation in the isolated kidney as suggested by Shipley and Study (1951) This finding is in contrast to observations from kidneys in situ by Thurau and Wober (1962) and Thurau (1964 a) here kidneys were claimed to be denervated surgically In these in situ experiments high systemic blood pressures were obtained either by bilateral carotid occlusion or by procaine injection into both vagal nerves and the walls of the carotid sinus (neurogenic hypertension) It is not stated however whether the renal artery had been divided and later

reconnected (the only explicit way of denervation) or whether it had been stripped only Any changes in the proximal intratubular or peritubular capillary pressures of kidneys in situ were not observed when the systemic pressure changed within the range of autoregulation Whether solely myogenic or possible neurogenic or intrarenal humoral substances with vasoconstrictor and/or tubular effects are involved in these latter experimental conditions, the most reasonable general interpretation of the unchanged glomerular filtration rate seems to be that under conditions in which the tissue pressure increased in response to the elevation of the perfusion pressure the glomerular propulsive pressure as well as the intratubular pressure had increased in parallel, whereas neither the glomerular propulsive pressure nor the proximal intratubular pressure had changed significantly under the in situ conditions Thus the effective filtration pressure has not changed significantly in any of these situations As emphasized also by Thurau (1964 b) any detailed interpretation of the possible mechanisms involved in this phenomenon cannot be expected until aspects of the tubular reabsorptive function and hydro dynamic resistances to luminal flow are included in the discussion

As apparent from the above discussion the current and conventional theory of the mechanisms involved in urine formation, especially those concerning the renal handling of salt and water is based upon a complex of interrelated assumptions In these assumptions the absence of direct experimental evidence is conspicuous Thus any evidence in support of the first assumption has never been provided (clearance of inulin a measure of glomerular vascular factors only), on the contrary gross disturbances in the tubular function suggest that this assumption may be untenable The second assumption (load dependency of proximal sodium reabsorption) has not been and cannot be established by the clearance technique as usually performed since rapid sequence of events cannot be studied by this method Conventional micro puncture investigations have not been designed for this type of study either and data thus obtained have not been analysed with special reference to verify this assumption Finally direct observations have excluded the validity of the *assumptions III IV and V Therefore it seems rather pretentious* when Homer Smith in his introduction to The Kidney (1951 p XXII) states in an authoritative way Renal physiology has now passed into a quantitative phase where unsupported speculation and empirical description are no longer warranted *It might rather be reasonable now to declare that there is no reason whatever for a maintenance without discussion of this theory* More approaches to direct experimental confirmation are highly desirable and as will be seen also available

Before proceding to the discussion of the experimental data which may disclose whether the major fraction of the filtered water and solutes is reabsorbed along the proximal tubular segment at a rate, which may be characterized as load dependent or load *independent* (i e Tm limited) it may be of advantage tentatively to have the process(es) of trancellular transport in the mammalian proximal tubules characterized at the cellular level Such characterization, which now seems possible on the basis of a series of the most recent investigations using direct methods may add to our present understanding or concept of the phenomenon of Tm limitation and gradient limitation of transcellular transports in the proximal tubules and thus will serve as foundation for the following discussion

CHAPTER II

Transcellular transport processes across the mammalian proximal tubule cell

In the foregoing chapter no clear distinction has been made between the vague term transfer of filtrate and transfer of salt or sodium The proximal reabsorption being an isosmotic reabsorption predominantly of sodium chloride, and bicarbonate ions and water and since it is known as discussed in the previous chapter (Ch I B c p 19–20), that sodium is actively reabsorbed against an electrical potential gradient the water following passively (osmotically), a preliminary quantitative description of transcellular fluid transfer therefore as a first approximation concerns primarily the uphill transport of sodium ions

A RATE LIMITATION OF ACTIVE TRANSPORT IN GENERAL

In attempts to characterize formally and experimentally an active transport process across biological membranes as opposed to diffusion a thermodynamic distinction has been preferred e g by Rosenberg (1948) and by Ussing (1949 c) Accordingly the term active transport whenever used in this review is limited to processes of transfer of chemical matter from a lower to a higher chemical or electrochemical potential (uphill transport), processes requiring energy generally by coupling to metabolic energy yielding processes of the cell It should be emphasized that as a fundamental basis for the entire discussion of active transport phenomena the general thermodynamic functions are assumed to be well defined in the actual biological compartments or phases considered a postulate which well may be questioned, especially as regards systems far from a state of equilibrium

The derivation from Fick s law of Ussing s flux ratio equation

(1) $$\frac{M_1}{M} = \frac{C_1}{C}$$

has been of the greatest importance and of the utmost practical value for the design of the experimental approaches in this equation M_1 is the

unidirectional flux in the direction (1→2) and M_2 the inversely directed flux and C_1 and C_2 the concentrations of substance on the two sides of the membrane In the case of charged particles (ions) a formally identical relationship was derived by Ussing (1949 b)

(2) $$\frac{M_1}{M} = \frac{a_1}{a}$$

expressing that the flux ratio is equal to the ratio between the electro chemical activities of the ion on the two sides of the membrane when the ion moves passively in the uncombined state through a membrane where it is not subject to solvent drag Thus in the case of insignificant net water transfer an active net transport of e g sodium ions is generally indicated by a flux ratio exceeding the one calculated from the equation (2) The usual procedure in the attempt to characterize active transcellular sodium transport is, by approximation to consider the epithelial cell layer(s) as one single membrane, and further to define a quantity, E_{Na} which is denoted as the active transport potential of sodium or the driving force of the sodium pump referring to an idealized system in which there is no electrical gradient nor any concentration gradient across the membrane Such system may apparently be experimentally realized in a variety of epithelial tissues (e g the frog skin and the toad bladder), in which the normal electrical potential difference across the membrane is abolished by application of a short circuit current and in which identical sodium concentrations on the two sides of the membrane are established e g by bathing the tissue in Ringer solutions of identical ion composition on both sides In this case we have

(3) $$E_{Na} = \frac{RT}{F} \ln \left(\frac{M_1}{M}\right),$$

in which R denotes the gas constant, T the absolute temperature and F the Faraday number The fluxes M_1 and M_2 may be determined directly by the double isotope tracer method It is obvious that the rate of net sodium transfer through the membrane may be influenced by an opposed electro chemical potential possibly created by the active transport itself and will reach zero value when the electrochemical potential, *against which sodium is forced* approaches the E_N In this case the active transport of sodium itself creates a limiting potential gradient through the membrane and the net transfer rate may be characterized as gradient limited The diuretic effect of e g mannitol loadings seems to be due predominantly to such an effect on the proximal reabsorption rate The presence in the proximal fluid of the non reabsorbable but osmotically active mannitol will prevent water

from following the actively transferred sodium ions The luminal fluid sodium concentrations is thereby reduced to values lower than those in the peritubular plasma, and the active transport now proceeds not only against the electrical potential of 20–25 mV but also against a chemical potential As demonstrated by Windhager and Giebisch (1961 a and b) the rate limiting sodium concentration difference in proximal tubules of rats was 40–60 meq/l If the sodium concentration in the luminal fluid was reduced to establish a concentration difference exceeding this value the direction of net sodium flux was reversed

As concluded by Ussing (1949 b) Deviations from the flux ratio equation indicate that the ion does not diffuse in the free state only but in part at least as a component of some other moving particle in the membrane The term carrier is usually applied to such other moving particles in the membrane with which the transported matter is coupled in complex formation Although hypothetical in nature an extensive body of evidence now supports the carrier concept, which has provided a highly useful means by which to explain the qualitative and quantitative relations of several examples of transport in biological systems, as stated by Wilbrandt and Rosenberg (1961) The equation common to all types of carrier transfer relates the transfer rate (v) to the movement of the carrier substrate complex in the membrane

(a) $$v = D' (CS_1 - CS)$$

in which D' is a permeability constant (the diffusion coefficient of the transported matter in the particular membrane of a certain thickness), and CS_1 and CS are the concentrations of carrier substrate complex on the two sides of the membrane respectively

Quite generally the carrier system is characterized by the following steps The binding between substrate and carrier the diffusion of the complex formed and the splitting of the complex into substrate and carrier on the other side of the membrane The kinetics of this transport of substrate will depend on the rate constants of these steps In the most simple case in which the substrate (S) is assumed to be in equilibrium with the carrier (D less than the rate constants of the reactions) and in which the further assumption is made that the diffusion constant of the free carrier is identical to that of the carrier substrate complex, the kinetics may be described by the equation

(b) $$v = D\, C_t \left(\frac{S_1}{S_1 + K_m} - \frac{S}{S + K_m}\right)$$

in which C_t denotes the total concentration of carrier and K_m the dissociation constant of the carrier substrate complex (the Michaelis constant) It appears

from the equation (b) that the rate of transport will increase with increasing substrate concentration on one side of the membrane and since the last term the saturation term cannot exceed the numerical value of 1 $D C_t$ will indicate the theoretical maximum transport rate and may be substituted in the equation by V_{max}

(c)
$$v = V_{max} \left(\frac{S_1}{S_1 + K_m} - \frac{S}{S + K_m} \right)$$

Thus D C or V_{max} is a capacity term of the carrier kinetics

According to the treatment of carrier kinetics in systems capable of uphill transport given by Wilbrandt and Rosenberg (1961) one of the possibilities of active carrier transport may be realized when the transport is coupled to the metabolic reactions which may change the K_m value to a different extent on the two sides of the membrane In this case we have

(d)
$$v = V_{max} \left(\frac{S_1}{S_1 + K_1} - \frac{S}{S + K} \right)$$

If $K \gg K_1$ S will move uphill in the direction of ($1 \rightarrow 2$) until a steady state is reached in which

$$\frac{K_1}{K} = \frac{S_1}{S}$$

Again we obtain an increasing transport rate with increasing substrate concentration on one side (S_1) and in the non steady state in which no rate limiting potential gradient is created we may reach a maximum transport rate at the saturation level at which further increments in the substrate concentration no longer influences the rate If this is the normal condition in the case of proximal sodium reabsorption the rate of sodium transfer may be characterized as *Tm limited*

In the case of active transcellular net transport the situation obviously is far more complicated theoretically as well as experimentally since the transported matter has to move across at least two cell membranes which apparently must be quite differently characterized among other things as regards properties of permeability and capability of uphill transport Hence a fairly detailed characterization of each of the bounding cell membranes and of the transport across these is necessary in order to explain quantitatively the overall net transfer Besides when the discussion aims at an understanding at the cellular level of Tm limitation and gradient limitation of net transfer rates and a possible regulation of these rates it is equally necessary to be able to decide whether one or the other of the two cell boundaries constitutes the rate limiting step in the transcellular transport

B THE RATE LIMITING STEP IN ACTIVE TRANSEPITHELIAL ABSORPTION

Since active sodium transport across epithelial cells, which according to Ussing (1960) apparently is a characteristic of all animal cells which absorb sodium and chloride from the outside into the organism generally is considered to be due to essentially identical mechanisms in the tissues, in which it is established such as the frog skin the urinary bladder of the toad the intestinal epithelium of the toad and guinea pig and kidney tubules it may be appropriate briefly to draw into the discussion first some data and current hypotheses derived from observations on the most carefully studied tissues, the frog skin and the toad bladder Thus, it was shown by Koefoed Johnsen and Ussing (1958) that the electrical potential difference across surviving frog skins, which were treated either with copper ions in order to minimize the chloride permeability or were bathed in Na SO_4 Ringer (sulphate being non penetrating), was almost linearly related to the logarithm of the potassium concentration of the inside solution and with a nearly correct slope for a potassium selective electrode Further it could be demonstrated that practically all exchange of potassium takes place between the cell interior and the inside solution, and that the inward facing membrane permeability of free sodium ions is very low On the other hand, when the skins were copper treated or, even better, bathed in sulphate Ringers the potential increased almost linearly with the logarithm of the outside sodium concentration imitating the characteristics of a sodium selective electrode It was concluded, therefore that the outward facing membrane in the absence of penetrating anions behaves as a sodium electrode [selectively permeable to sodium (– and lithium) ions] while the inward facing membrane behaves as a potassium electrode Based on these observations the electrical potential difference across the living skin epithelium could be explained, if an active transport mechanism (a pump) were localized at the inner border which might force sodium ions from the cellular cytoplasm into the inside bathing medium and potassium from the inside medium into the cellular cytoplasm The pump is believed to keep the intracellular sodium concentration low and fairly constant thereby creating an electrochemical potential gradient for diffusion of sodium from the outside solution into the cell, this would give rise to a sodium diffusion potential making the outside electrically negative to the cell At the inner membrane potassium is assumed to move in the steady state in a closed circuit being actively transferred into the cell and diffusing from the cellular cytoplasm into the inside solution thereby giving rise to a second diffusion potential which makes the inside solution elec

trically positive in relation to the cell In this way a specific active sodium transfer might be established and the electrical potential explained

While the nature of the electrical potential still is intensively debated and probably may be of different types under different conditions and in different tissues attention should be drawn to two other essential features of the active transcellular sodium transport, implied in Ussing s model which seem to be generally accepted for all tissues investigated constituting a fundamental part of the hypothesis upon which all subsequent models proposed for each particular type of cells is based *1) The pump mechanism is assumed to be supplied with sodium ions from the intracellular cytoplasmatic sodium pool and 2) the intracellular sodium concentration is assumed to be maintained low by the same pump mechanism which effectuates the active transcellular net transport*

a) *The rate limiting step of the sodium transport across amphibian skin and urinary bladder*

These latter assumptions which imply that sodium ions enter the cell passively down an electrochemical potential gradient may have some bearing upon the question of the rate limiting step of net sodium transport

In the frog skin the rate of net sodium transport (outside→inside) was found to increase with increasing outside sodium concentration in the range from 1 to about 110 mmol NaCl although not in a linear proportion (Ussing 1949 a) With increasing sodium concentration the relative increase in net flux diminished in a way suggesting that the transfer asymptotically ap proaches a limiting maximum rate Thus the transport process apparently shows saturation kinetics The same type of kinetics was demonstrated by Frazier Dempsey and Leaf (1962) in the case of sodium transfer across the toad bladder in which a transfer maximum, evaluated on the basis of the short circuit current was reached at an outside (mucosal side) concen tration of about 60 mmol NaCl Further their results indicated that the mucosal entry of sodium represented the limiting step of the rate of transfer since the tissue sodium pool, initially increasing with increasing sodium con centration in the mucosal medium also levelled off at a concentration of about 60 mmol NaCl as should be expected only if the mucosal entry of sodium was limiting the rate of net transfer Altogether these findings indicate that sodium entry across the mucosal membrane although possibly passive involves some considerable interaction with the membrane or some constituent thereof (a carrier) Finally the stimulating effect of vasopressin on the active sodium transport (short circuit current) was also localized to the mucosal

membrane, as indicated by an invariable increase in the tissue sodium pool in the presence of vasopressin Recently Sharp and Leaf (1963) reported that the tissue sodium pool of the toad bladder also had increased during aldosterone stimulation of the active sodium transport

In the frog skin severe interference with the sodium pump, as indicated by a rapid drop in the electrical potential e g by oaubain treatment or a lowering of the pH of the inside bathing solution to 5 9, was not followed by a cellular swelling as might have been expected (MacRobbie and Ussing 1961), indicating a tighhtening both of the outward and inward facing membrane to ions A low pH in the inside medium also reduced the water permeability of the inside membrane to about 50 per cent of the value at pH 8, and the authors conclude 'It may be that any interference with the pump mechanism is accompanied by a decrease in the passive permeabilities to ions and that the active ion transport and the passive fluxes are not entirely independent

If it were justified to generalize from these results obtained from some amphibian epithelia, which actually have far more resemblance to the mammalian distal tubular epithelium than to the proximal epithelium functionally as well as morphologically, it might *a priori be expected or suggested that the rate of reabsorption of filtrate in the proximal tubules of the mammalian kidney (high outside (luminal) sodium concentration) were also limited by the transfer process at the luminal brush border membrane (morphologically the outward facing border)*

b) *The rate limiting step of the mammalian proximal tubular reabsorption of salt and water*

No direct or unequivocal evidence has yet established whether the luminal or the peritubular membrane of the proximal tubule cell constitutes the rate limiting step of reabsorption Some indirect evidence, apparently suggesting that the access of filtrate to the luminal brush border of the proximal cells might be of importance for the pump was believed to support the contention that the luminal process of entry of ions to the cell is rate limiting (Leyssac 1965 a) It was observed in rats that as soon as the reabsorption of proximal luminal fluid was completed after interruption of the renal blood circulation and filtration a significant swelling of the proximal cells occurred Since it was observed also that proximal cells of rat kidney cortical slices did not gain their original volume, but on the contrary swelled even more if transferred to oxygenated Ringer solutions or plasma, it was inferred that lack of oxygen probably could not be the only reason for the observed swelling (by

interference with the pump mechanism) and that the lack of luminal fluid (substrate) in part at least might be of importance for the swelling phenomenon However later investigations (unpublished) disclosed that the sodium concentration of similar cortex slices incubated in oxygenated Ringer solution depends on the incubating temperature and the efficiency of stirring Tissue sodium concentration might be lowered by as much as 10–15 meq per kg of wet weight when slices were incubated either at 25°C as opposed to 38°C or under violent stirring with continuous supply of oxygen The secondary swelling seen after incubation of slices in oxygenated Ringer without such stirring may therefore most likely be due to lack of oxygen no valid arguments were provided which could possibly exclude that lack of oxygen might also be the only cause of the initial (or primary) swelling seen some 25 sec after interruption of the renal blood circulation Thus the question may be regarded as completely unanswered

A different approach to the problem may be an attempt to sum up data characterizing the individual transfer processes of each particular component of the reabsorbate across each of the two bording membranes and evaluate these observations in relation to the rates of net flux The prerequisite of the possibility of obtaining some insight into the characteristics of ion transports across the luminal and peritubular membrane has been the collection of data concerning the direction of net movement of the given ion species estimates of respective ion concentrations on the two sides of the membrane and of the electrical potential profile of the proximal tubular cell Direct measurements under free flow conditions and in the absence of osmotic diuretics of the electrical potential profile of the mammalian proximal cell not being available as far as I know reference shall be made to that of proximal cells in Necturus in accordance with general practice (cf Giebisch and Windhager 1964) This seems to be justified for the following reasons First Marsh Ullrich and Rumrich (1963) have reported an electrical potential difference across the proximal peritubular membrane in rats of about 60 mV (cell negative) under microperfusion conditions of almost zero net fluid flux (cf below) Whittembury (1964 1965) found similar values across the peritubular membrane of guinea pig cortical cells in slices in which the tubular lumina are known to be completely occluded (Bojesen and Leyssac 1965) for which reason a net transport hardly can take place Second almost all essential features of the mammalian proximal reabsorption are similar in proximal tubules of Necturus kidneys Thus as in mammals the proximal tubular reabsorbate is isosmotic with Necturus plasma (Windhager et al 1959) the water reabsorption is passive and due almost entirely to the reabsorption of sodium chloride (cf Ch I p 20) In both kidneys a transtubular electrical potential

difference of about 20 mV exists across the proximal tubular wall (lumen negative) (cf Ch I, p 20) Further, taking into account that the measurements were performed in different series of experiments using the same animal species, a fairly good agreement was obtained between the short circuit current in Necturi kidneys (corresponding to an active monovalent ion transport of $4\,6 \times 10^{-11}$ Eq/cm²sec Eigler 1961) and the net sodium flux of $6\,2 \times 10^{-11}$ Eq/cm sec as estimated from radioisotope measurements by the stopped flow microperfusion technique (Oken et al 1959) In the rat a similar close agreement between these two methods of estimating the active net sodium transport was obtained (the short circuit current corresponding to a net flux of 34×10^{-10} Eq/cm sec, as opposed to the net sodium flux of 30×10^{-10} Eq/cm²sec) as shown by Windhager and Giebisch (1961 a) A peritubular potential difference of 70–74 mV (cell negative) in the proximal cells of mammalian tubules under physiological free flow conditions as measured directly in Necturi proximal cells e g by Whittembury (1960), therefore may be accepted as the most likely value With a transtubular electrical potential difference of 20 mV (lumen negative) a potential difference of 50–54 mV (cell negative) across the luminal brush border is calculated

The application of the microperfusion technique described by Gertz (1963) has been a particularly suitable approach to a characterization of ion transports across the two cell borders of mammalian proximal cells, by this technique a condition of zero net flux may be approached by the introduction in the single proximal lumen of a slowly penetrating non electrolyte such as raffinose In brief, an isotonic aqueous raffinose solution is injected intraluminally between two oil columns After an initial volume increase of the interposed aqueous solution due to influx of sodium potassium and chloride and accompanying water, the volume increase ceases again after about 50 sec to be followed by a slow and exponential volume decrease (Gertz 1963), during which the concentrations of sodium, potassium and chloride remain constant according to Kashgarian et al (1963) During this latter condition of exponential volume decrease the net fluid outflux is reduced to about 15–20 per cent of normal it may be considered as an approximation to a steady state condition as regards the rapidly permeating ions Under this experimental condition measurements of the transtubular electrical potential difference (between the isolated fluid column and the interstitium) of the concentrations of sodium potassium, and chloride in plasma and in the simultaneously recollected perfusion solution permitted an evaluation of the transtubular electrochemical potential differences of each ion species

During the steady state condition with raffinose in the lumen the proximal transtubular electrical potential difference in rats amounted to an average of 24 mV (lumen negative), as opposed to 20 mV during free flow conditions The mean sodium concentration was 149 meq/l in the plasma and 109 meq/l in the perfusion fluid (corresponding to 103 meq/l after correction to a state of absolutely zero net flux), giving a tubular fluid to plasma sodium concentration ratio (F/P Na) of 0 7 (Kashgarian et al 1963) From these data the active transcellular transport potential for sodium (E_{Na}) was estimated from the equation given by Ussing (1960, p 121)

$$E_{Na} = \frac{RT}{F} \ln \left(\frac{c_i}{c_o}\right) + E + \frac{RT}{F} \ln \left(\frac{M_i}{M_o}\right)$$

(in which c_i and c_o denotes the peritubular and luminal concentrations respectively and M_i and M_o the unidirectional fluxes M_i indicating the direction of flux from the lumen to the peritubular interstitium) Since the net flux may be considered as approaching zero value the last term of the equation also becomes approximately zero The proximal E_{Na} then was found to be 31 mV

As mentioned above (p 31) an electrical potential difference of 60 mV (cell negative) across the peritubular membrane was observed by Marsch Ullrich and Rumrich (1963) under such conditions of approximately zero net fluid flux from which it follows that the luminal membrane potential is about 35 mV (cell negative) Since it is known also that the intracellular sodium concentration is considerably lower than that of proximal fluid and plasma (as confirmed by Bojesen and Leyssac 1965 cf below) the active element of the transport process might be expected to be located at the peri tubular end of the cell according to the general concept that sodium is pumped out from a homogeneous cytoplasmatic pool On the same assump tions it is inferred that sodium entry across the luminal brush border occurs passively down a considerable electrochemical potential gradient But ap parently sodium ions are not transported across this membrane by simple free diffusion as pointed out by Ullrich (Marsh Ullrich and Rumrich 1963) If they were an electrochemical equilibrium across the luminal membrane should be approximated under the experimental steady state condition of approximately zero net flux Quite obviously such equilibrium was very far from being approached The intracellular sodium concentration in the equil ibrium state at a luminal sodium concentration of 110 meq/l and an elec trical potential difference of 35 mV (cell negative) would be of an order of magnitude of 500–600 meq/l Such concentration of sodium in the cyto

plasmatic pool is clearly incompatible with the idea of isosmolality of the tubular and intracellular fluids which seems generally accepted as well as with the general notion of a high intracellular potassium to sodium concentration ratio The authors were therefore driven to conclude, that sodium ions were restricted from reaching equilibrium, a fact which might be explained by an active transport of sodium from the cell also into the proximal lumen The opposite direction of net sodium flux into the cell was still believed to be due to free diffusion It might be questioned whether or not the lack of equilibrium across the luminal membrane during the steady state conditions could be due to a low sodium permeability This however does not seem very likely taking into account the extremely large net flux of sodium which normally takes place across the cellular wall as a result of still much larger fluxes in each direction

Using the same method these investigators showed that potassium also is actively transferred across the proximal tubular cell Under the stationary microperfusion condition a mean proximal F/P K of 0 91 was observed With a transtubular electrical potential difference of 24 mV an active transtubular electrochemical transport potential (E_K) of 26 mV was found On the same general assumptions as in the case of sodium transport the high intracellular potassium concentration would necessarily indicate that potassium ions are actively translocated at least across the luminal membrane The further movement of this ion species from the cell into the peritubular interstitium might then occur downhill by free diffusion and/or by some type of carrier mediated transport mechanism As stated by the authors no data contradict even the possibility that potassium also is pumped actively into the cell on the peritubular side (morphologically the inside) as generally believed to be the case e g in amphibian epithelia finally they proposed the hypothesis that an active linked sodium potassium exchange mechanism operates at all cell surfaces of the proximal tubular cell They admit that such apparent transport symmetry obviously offers serious difficulties for a comprehension of the net orientation of this transport activity

This problem seems even more complex when the chloride transport mechanism is included in the considerations Thus although the overall proximal tubular chloride reabsorption apparently is of passive nature as the lumen is electrically negative in relation to the peritubular interstitium and as the luminal chloride concentration under free flow conditions can significantly exceed that of plasma a simple diffusion of chloride as the sole mode of transport probably is an inadquate explanation of the transfer mechanism which has been emphasized also by Giebisch and Windhager (1964) This statement is based on data presented by Kashgarian et al (1963),

obtained during the stationary microperfusion condition with raffinose in the luminal solution In the steady state (the phase of exponential volume decrease) a mean F/P Cl of 0 97 was found (when plasma values were corrected for water content and Donnan effect) The observation of a transtubular electrical potential difference of 24 mV (lumen negative) under this condition approaching zero net flux permitted the conclusion that electrochemical equilibrium for chloride was not established but a force of 23 mV seemed to oppose the luminal entry of chloride into the cell which would correspond to an active transport of chloride from the cell into the lumen with an E_{Cl} of 23 mV (lumen negative)

In the case of urea a purely passive mode of reabsorption was suggested by Rehberg (1926 b) on the basis of clearance studies in man, as previously mentioned (Ch I, p 14) Although this postulate apparently has been supported by numerous similar studies in a great variety of mammals some doubt about this general statement has been raised, since it was observed that urea clearance in the kangaroo rat and the white rat might vary independently of the simultaneous inulin – or truly endogeneous creatinine clearance and that occasionally the urea clearance in the kangaroo rat might exceed that of inulin (B Schmidt Nielsen 1955) Since a renal urea synthesis cannot be excluded and since it cannot be excluded either that the urea clearance might have exceeded the inulin clearance because of an excretion of urea deposited in a concentrated papillary interstitium at an earlier phase in addition to that filtered no unequivocal demonstration of active transport of urea in the mammalian nephron has been presented yet neither with respect to secretion nor to absorption It must be admitted, however as also emphasized by Gottschalk (1964 b), that the available data have not allowed any clear delineation of the character of urea movements in each of the nephron segments In the proximal convoluted segment in which about 50–60 per cent of filtered urea is reabsorbed the tubular fluid concentration of urea invariably exceeds that of plasma on an average by a factor of 1 5 as shown directly in micropuncture studies (Lassiter Gottschalk and Mylle 1961, Ullrich et al 1963) Thus if it may be assumed that the urea concentration in the cortical peritubular interstitial fluid is close to that in the peripheral plasma is seems safe to state that conditions apparently favour a passive overall transfer process at least in this segment of the nephron The lack of direct knowledge of intracellular urea concentrations in the proximal tubular cell excludes an evaluation of the individual transfer mechanisms across the two bounding membranes

Considered collectively these data present apparently a rather confusing complex of forces of various magnitudes favouring as well as opposing the net

movements of solutes and solvents from the proximal lumen into the peri tubular interstitium Actually each one of the new results seems to add to the confusion instead of completing a mosaic, in which a certain pattern was surmised In our attempts to describe quantitatively the net transport of water and solutes acros the proximal cells, in accordance also with the above mentioned observations, it is tempting to look for supplementary ideas, possibly obtainable by a comparison of the individual rates of net transport

As referred to previously in this review (Ch I B c), it has been esta blished by micropuncture studies that about 70–75 per cent of filtered water and sodium is reabsorbed at the end of the accessible part of the proximal convolution (60–70 per cent of the total proximal tubule length) as indicated by an increase in the F/P inulin to a value of about 3 at this site with a maintained F/P sodium of unity At this site 65–70 per cent of filtered chloride has also been reabsorbed Windhager and Giebisch (1961 b) measured tubular and plasma chloride concentrations in rats during infusion of a 0 9 % sodium chloride solution and obtained proximal fluid to plasma ratios only slightly above unity (mean 1 03) independent of the distance from the glomerulus Under antidiuretic free flow conditions a mean F/P Cl of 1 24 was found by Kashgarian et al (1963), but also in this report it appears that this ratio neither increases nor decreases along the proximal convolution Thus chloride is reabsorbed along with sodium and water in the same proportion as in the tubular fluid As mentioned previously (Ch I B c, p 23) this is also true for urea

Recent results of proximal tubular fluid and plasma concentrations of potassium in antidiuretic rats have shown that the tubular fluid concentration is reduced to values below the plasma concentration before the fluid has reached 15–20 per cent of the proximal length and then maintains an unchanged F/p K of an average of 0 8 (0 79±0 03 (SEM) throughout the convoluted part (Marsh, Ullrich and Rumrich 1963) Similar values were reported by Malnic Klose and Giebisch (1964) in control rats the ratio ranging from 0 69 to 0 91 in this report In rats maintained on a low potas sium diet this ratio was slightly higher, many value being close to unity (range 0 69 to 1 00) Also here the F/P K remained unchanged along the proximal convolution in both groups of animals indicating that about 70 per cent of filtered potassium is also reabsorbed when the tubular fluid has reached about 65 per cent of the proximal length and that potassium ions are also reabsorbed together with sodium, chloride and water in the same proportion as in the proximal fluid These recent investigations have confirmed in main the earlier report by Lichtfield and Bott (1962) in which the

F/P K was maintained close to unity along the proximal convolution in non diuretic rats

Finally it shall be mentioned that similar observations have been made with bicarbonate reabsorption in the proximal convoluted tubules of anti diuretic and water diuretic dogs (Clapp Watson and Berliner 1963) The mean bicarbonate concentration in the fluid collected from the proximal tubules was 16 to 17 meq/l in anti-diuresis and water diuresis respectively slightly less than the plasma concentration of 22 meq/l As in the case of sodium chloride potassium and urea the bicarbonate concentration showed no tendency to diminish (or increase) as fluid passed along this tubular seg ment either in anti diuresis or in water diuresis At the end of the accessible part about 70 per cent of filtered water, as inferred from the measured inulin ratios and about 75 per cent of the filtered bicarbonate had been reabsorbed The capacity of bicarbonate reabsorption, however seems to depend to a considerable degree on the experimental conditions and thus may present special and more complex problems than proximal reabsorption of sodium, potassium and chloride Rector, Bloomer and Seldin (1964) showed in normal anti diuretic rats that the proximal bicarbonate concentration decreased significantly along the proximal convoluted tubules as opposed to the above mentioned findings in dogs Although the degree of alkalosis in rats given $NaHCO_3$ infusions and in hypokalaemic rats was comparable every sample of proximal fluid in rats receiving $NaHCO_3$ had higher HCO_3 concentration than plasma and the tubular fluid concentration was increasing along this segment whereas every proximal sample from hypokalaemic rats had a lower HCO_3 concentration than plasma and it was decreasing along the convolution Any change in the proximal bicarbonate concentration could not be demonstrated however in samples collected from the same tubule (middle third of the convolution) before and after aortic constriction, which depressed the inulin clearance by 50–60 per cent On the assumption that the control clearance values have represented a certain part at least of the spontaneous range of variation it may be concluded that the proximal water and sodium reabsorption rate has been significantly depressed following the aortic constriction Thus the above observation that the tubular fluid bicarbonate concentration failed to change seems to indicate that the rate of bicarbonate reabsorption has been depressed to the same degree as the water and sodium reabsorption

It is apparent from these directly measured data *that the rather stereotyped proximal reabsorption obviously has the main character of a bulk reabsorption of an aqueous solution of electrolytes and urea* (of an ideal fluid) which

fact may seem rather peculiar considering the different characteristics of the individual processes and different forces acting on each one of the solute species at the luminal and peritubular membrane

The fact that variations in the rate of sodium and water reabsorption apparently are followed by parellel variations in the rate of chloride, potassium, bicarbonate, and urea reabsorption may seem even more peculiar Imagining that e g the potassium reabsorption rate were constant and independent of the sodium and water reabsorption, physiological variations in the rate of reabsorption of the latter, by which the rate of filtration not only of sodium and water but also of the other constituents of the ultrafiltrate is changed in parallel, would change the fractional reabsorbtion of potassium (in relation to rate of filtration) Such induced difference in the fractional potassium reabsorption relative to that of water would be reflected in a change in the F/P concentration ratios at the end of the proximal convolution, which then might explain the observed scatter of these ratios If however this were a valid explanation of this scatter, a significant increase in the scatter might be expected along the length of the proximal tubule Such tendency is not established by the many reported data on potassium transfer, and in the case of bicarbonate the above mentioned evidence has suggested that the bicarbonate reabsorption rate does change in parallel with that of sodium and water It therefore seems probable that *variations in the rate of proximal fluid reabsorption actually are variations in transfer rate of a volume of an aqueous solution of electrolytes and urea*

This picture of the transcellular transport across the proximal tubular cell however, seems rather contradictory in many respects if considered from a quantitative point of view Thus, the fairly close accordance between proximal net sodium flux as evaluated from the short circuit current and results obtained from microperfusion experiments makes it less likely that an active transport also of potassium and chloride participates significantly in the establishment of the total electrical current The quantitative relationship might possibly be accounted for at the peritubular membrane of the cell, across which only sodium appears to be actively forced into the interstitial fluid with chloride and potassium ions moving downhill, and that the difficulties involved in the quantitative description arise at the luminal border Furthermore other difficulties may be added

Ullrich Rumrich and Fuchs (1964) measured quantitatively the water permeability of the proximal tubular wall in rats using intraluminal infusions at constant rate of solutions containing sodium chloride at equilibrium concentration (the concentration at which net sodium chloride flux is zero in the presence of a non penetrating solute = 110 mmol NaCl) which had been made

hypo or hypertonic by addition either of raffinose or of mannitol Aliquots of the infused solution were subsequently withdrawn at a more distal site of the convolution and the length of the perfused segment measured in the nephron, which later was isolated by microdissection From the osmolalities of the recollected perfusion fluids and plasma samples, it was found that about 50 per cent osmotic equilibration was achieved after a flow along 200–400 μ of this segment (corresponding to a contact time of 0 3 to 0 6 sec), while complete equilibration was achieved at a perfused distance of about 1000 μ The same values were obtained for water influx and outflux When the data were plotted in a system with the logarithm of $\frac{(100-A)}{10}$ as the ordinate ((A) denoting the per cent osmotic equilibration) versus the length of the perfused segment as abscissa a linear relationship was found, the slope of which is a relative measure of the water permeability On the basis of this slope the luminal radius (used for calculation of the perfused area), the rate of infusion and the difference between the osmolality of the perfusion solution and plasma the coefficient of water permeability was calculated and found to be $17\,4\times10^{-8}$cm^3 cm^{-} sec^{-1} cm H_2O in the proximal convolution Based on the known rate of proximal fluid reabsorption during free flow and this permeability coefficent it was calculated that the proximal fluid osmolality should be lower by 23 mosm than that of plasma if the actual reabsorption of water were to be accounted for on the assumption that the water flux were due to an osmotic gradient created by active salt reabsorption as generally believed Since such difference in the osmotic pressure across the proximal tubular wall does not exist under free flow conditions their conclusion viz *that the water permeability actually is too low to account for the reabsorption of water* seems justified consequently the transcellular water movement was postulated to occur, at least in part on account of hydrodynamic flow in subcellular compartments (channels) The space between the infoldings of the peritubular plasma membrane (the β cytomembrane) was believed to constitute the morphological equivalent of these micro channels

Finally attention shall be drawn to the estimation of the proximal intra cellular cation concentrations as calculated by Bojesen and Leyssac (1965) on the basis of analyses of sodium and potassium content in weighed fragments of rat cortical tissue frozen at the moment of removal On the basis of the measured plasma space and the calculated proximal intraluminal volume the total content of extracellular sodium and potassium could be calculated and when subtracted from the measured total content in the tissue the intra cellular sodium and potassium content could be calculated as the difference

The intracellular concentrations were found to be 5–15 meq Na^+/kg of cellular water and 125–135 meq K^+/kg of cellular water This result may be compared with the one derived from an indirect approach by which the efflux curves of labelled sodium (^{22}Na) from incubated rat cortical cylinders were subjected to graphical as well as numerical analysis, based on two different two compartment models, a non communicating and the more realistic communicating two compartment model respectively (Bojesen, Leyssac and Svejgaard Nielsen 1965) A fairly close agreement between the results of both types of analysis was obtained and a remarkably good fit with directly recorded experimental data was obtained by the numerical analysis From the latter it appeared that about 80–85 per cent of exchangeable tissue sodium was in diffusion equilibrium with the incubating medium the calculated virtual diffusion coefficient being 7.3×10^{-6} $cm^2 sec^{-1}$ (25°C) From the separately measured concentration of tissue sodium in similar cylinders relative to that of the incubation medium it was calculated that the remaining 15–20 per cent fraction of exchangeable sodium, which was not in diffusion equilibrium with the medium, occupied a fraction of the total tissue water of 30–45 per cent The sodium concentration in this compartment, representing predominantly the proximal intracellular space of the isolated tissue was found to range between 35 to 55 meq/l which is considerably higher than the concentration calculated on the basis of analyses of cortical tissue frozen in vivo It seems most likely, however, that the intracellular sodium concentration in cortical tissue incubated in vitro is somewhat higher than in the in vivo state This statement is in agreement with the finding by Whittembury (1964, 1965) that the electrical potential difference across the peritubular membrane in cortical slices of a mammalian kidney was about 10–30 mV lower than values obtained from kidneys in situ (cf p 35) and also with the observation by Leyssac (1965 a) that the proximal cells were swollen some 25–30 sec after removal of the kidney, at which time the proximal lumina are occluded Based on these arguments it seems fairly safe to state that the intracellular sodium concentration of reabsorbing proximal cells in situ is very low at least lower than 30 meq/l This conclusion permitted a quantitative evaluation of the general assumption that transcellular transferred sodium ions are pumped actively from a homogeneous cytoplasmatic sodium pool across the peritubular membrane (Bojesen and Leyssac 1965) On the basis of a cellular sodium concentration of 14 meq/l, a plasma sodium concentration of 140 meq/l, and an electrical potential difference of 74 mV (cell negative) it was calculated the the minimum thermodynamic work required to accomplish the net transport of one equivalent of sodium would be 3200 cal at 37°C disregarding the (unknown) extra energy consumption involved

in order to overcome the internal resistance to sodium movement With a value of 25000–28000 cal corresponding to one equivalent of oxygen and 7–6 equivalents of sodium transported for each suprabasal equivalent of oxygen consumed in the mammalian kidney as found by several investigators (e g Deetjen and Kramer 1960, Lassen, Munck and Thaysen 1961), the efficiency will become 75–90 per cent Since furthermore the resistance has to be overcome and some additional entropy production must be accounted for when sodium ions are transported to a higher electrochemical potential at the peritubular side of the membrane *it seems fairly safe to conclude that the general assumption underlying the calculation is open to serious doubt* Hence an alternative assumption seems to be desirable as in the case of water movement it has been suggested that transcellularly transferred sodium is not mixed with the cytoplasmatic sodium pool, but is confined to intra cellular structures allowing a localized high intracellular sodium concen tration (Bojesen and Leyssac 1965)

In summary all these results have converged to the concept of a cytophysi ological mechanism in many respects similar to micropinocytosis being in volved in the transport of water and solutes across the proximal tubular cell, most likely being the physiological mechanism by which the tubular fluid with its dissolved constituents enter the cell from the tubular lumen This hypothetical concept seems to solve at least most of the quantitative problems which have accumulated pari passu with some data recently obtained by direct measurements These results, however do not prove that a luminal transport process is rate limiting to the proximal net transfer of water and electrolytes (and urea) nor do they indicate that this process exhibits a Tm limitation although this possibility certainly should be seriously considered and experi mentally investigated All of the results discussed in this review are apparently compatible with such hypothesis some even strongly support it Furthermore electron micrographs of mammalian proximal tubular cells fixed in the reabsorbing state of function prior to the luminal occlusion and cellular swelling have disclosed the existence of an axial structure in each microvillus of the luminal brush border which morphologically is resembling a channel of about 10 mμ (100 Å) in diameter (Hanssen and Herman 1962) When fixed in this state of function the brush border is separated only by a rather narrow apical cytoplasmatic layer from the infoldings of the peritubular plasma membrane as seen from the electron micrographs presented by Maunsbach Madden and Latta (1962) several microvesicles are found in this apical cytoplasm This finding further supports the hypothesis of a luminal micropinocytosis the central channels in microvilli and the microvesicles being the morphological equivalent of this physiological transport mechanism

Thus, according to the hypothesis proposed above, reabsorbed water and electrolytes (and urea) enter the proximal cells by a luminal micropinocytotic process and is moved in this way through the cell to its basal boundary, the infoldings of the plasma membrane or the basement membrane Across this latter boundary membrane the further transport is achieved by an active, energy requiring mechanism, probably a sodium pump of a type so far undetermined i e the reabsorption has the character of a two step trans cellular transport process It should be emphasized that this theory implies that the active element under physiological conditions is supplied with sodium at a high concentration equal to the concentration in the proximal luminal fluid and furthermore that the transcellular transport mechanism in this specialized cell type apparently is spatially separated from the active mechanism by which the cell – like all other cells in the organism – maintains its cytoplasmatic cation concentrations Naturally this does not exclude that these two active sodium transporting mechanisms may be accomplished by the same type of enzyme system such as an adenosin triphosphatase system as the one described by Skou (1957, 1964) In the presence of Mg^{++}, the activity of this enzyme system is characterized by its dependence on sodium and pot assium ions In the presence of potassium the enzyme activity increases with increasing sodium concentration, a plot of the effect of sodium concentration on the activity yielding a typical S shaped curve (Skou 1957) However, the maximum activity may be depressed by increasing the potassium concentration In the enzyme system isolated from crab nerves a maximum activity was obtained in the presence of 100 mM or more of sodium and 20 mM of potassium with the steep part of the curve in the range of 10–50 mM sodium Enzyme systems with similar main characteristics, located in subcellular particles have been isolated also from a large number of mammalian tissues including kidneys (e g Skou 1962) in which the affinities to sodium and potassium appear to be somewhat higher than in those prepared from crab nerve On the main assumption that this enzyme system forms part of the transport mechanism (an active linked Na^{+}–K^{+} transport), by which the mammalian cell maintains a high potassium low sodium concentration, it is believed that the steady state is maintained at the steep part of the curve relating enzyme activity to sodium concentration Thus if the intracellular sodium concentration tends to increase, the enzyme activity increases and con sesequently the active transport of sodium out of the cell increases Were this hypothesis to be confirmed eventually it still might remain compatible with an assumption of a similar system forming part of the active element in the transcellular transport mechanism If so and in accordance with the present hypothesis the enzyme system would operate under conditions of high sodium

low potassium concentrations (i e the concentrations in the micropinocytotic vesicles), at which the enzyme activity is at its maximum and uninfluenced by minor changes in the cation concentrations If the active element of the transport process actually were to operate under optimal conditions as in such hypothetical case (and in the absence of a limiting electrochemical gradient) the rate of net transport might well be limited and regulated by the rate of the micropinocytotic step

However, irrespective of the possible mechanisms of the active step in the over all process both steps in the transcellular transport may be rate limiting to the reabsorption, depending on the experimental situation If conditions are established favouring the achievement of a sufficiently steep electrochemical gradient the basal thermodynamically active step may become rate limiting to the process Then the rate of transfer is gradient limited as e g in mannitol diuresis during which the luminal sodium concentration becomes reduced The active peritubular mechanism is then supplied with a low concentration of sodium and a limiting electrochemical gradient may be created across the peritubular membrane Under other conditions in which such gradient is not established as under physiological conditions the luminal micropinocytosis may be the probable rate limiting step That the reabsorption process under these latter conditions acquires the character of a Tm limited (or capacity limited) process independent of the filtered load will be comprehensible The rate of micropinocytosis then determines the transport capacity and the kinetics of transport may be described in terms of carrier kinetics in which the luminal membrane with its adsorbed and/or enclosed solutes and solvent represents the carrier substrate complex proper (the moving particle of the cellular wall representing the membrane) (cf p 30–31) The extent to which the likeness with carrier kinetics is strictly tenable might reasonably be the subject of some further discussion

For the sake of simplification the discussion may be restricted to the proximal sodium transport Naturally the fact that changes in the filtered fluid volume (or amount of substrate) are without any influence per se on the transfer rate is not a problem The problem arises when the sodium concentration is experimentally elevated e g by hypertonic saline infusion

Two extremes among the possibilities may be considered

Possibility 1 The first possibility is that reabsorbed solutes are initially adsorbed (specifically) to the brush border membrane which subsequently by membrane flow carries the solutes into the cell by the infoldings (channel formation) at the tip of the microvilli At a certain membrane flow rate the rate of solute transfer (or sodium transfer in this particular case) will be determined by the number of adsorption sites when amounts of solute are

sufficient to saturate the membrane The accompanying water might be confined to the interspace between the membrane surfaces and in the micro vesicles, in which the solute concentration is rather insignificant This being the case an increased luminal sodium concentration (>140 meq/l) would not influence the rate of transfer (assuming unchanged membrane flow rate), since the membrane (or carrier') was already saturated at the lower concentration Thus the likeness with carrier kinetics seems perfect Above the saturation level the rate of transfer will be maximal, determined only by the capacity term – the rate of membrane flow and microchannel formation –, which indeed may be variable In this case it may be a matter of a true sodium Tm as well as of a volume Tm

Possibility II might be that reabsorbed solutes and water were unspecifically ingested by the microchannel and vesicle formation without 'adsorption to the membrane surfaces In this case the rate of transfer would also depend on the rate of ' micropinocytosis , or volume transport capacity and it would be justified to use the term volume Tm But the rate of sodium transfer would not be strictly load independent at least not at minor variations of the sodium concentration, on the contrary it would increase with increasing concentration The kinetics of sodium transport would deviate from saturation kinetics at or above the saturation level and it would not be strictly correct to use the term sodium Tm

Finally all the (more likely?) possibilities between these two extremes should be considered which on principle correspond to the second case with respect to sodium Tm Chapman Andresen and Holter (1964) have demonstrated in amoebae such a process which essentially is similar to the latter possibilities (those between the two extremes) They showed that labelled protein (albumin) could be ingested specifically by pinocytosis due to adsorption to the surface membrane By immersion of amoebae in a solution containing ^{131}J labelled albumin and ^{14}C labelled glucose under conditions of low temperature, at which pinocytosis does not occur, a comparison of the ratios between the two compounds in the immersion solution and adherent to the amoebae showed that protein was absorbed preferentially by the amoebae with an enrichment factor of 36 By the same procedure, carried out at room temperature at which pinocytosis is induced by the protein containing medium and comparison of the activity ratios between the solution and within the amoebae protein was found to have concentrated in the pinocytotic vesicle (or vacuole) with an enrichment factor' of 14 This observation allowed the conclusion that large amounts of protein, but insignificant amounts of glucose are first adsorbed to the cell while the fluid phase ingested consists of the bathing solution of protein and glucose

These considerations may have some bearing on the interpretation of certain data obtained in clearance and micropuncture studies It was observed in clearance studies on rabbits (Kruhoffer 1950, table VII and VIII) during hypertonic sodium chloride infusions and increasing plasma sodium concentration that the rate of filtration (inulin clearance) as well as the rate of sodium reabsorption increased initially At high plasma sodium concentrations a constant inulin clearance could be obtained within a certain range of increasing sodium concentrations By calculation of the amount of sodium (meq Na) reabsorbed per litre of filtrate formed it was observed that at a certain point the increase in sodium reabsorption rate ceases and at further increments in the plasma sodium concentration ($\gtrsim$180 meq/l), the rate of sodium reabsorption decreases with increasing sodium concentration at a constant rate of filtrate formation At these very high sodium levels there was a tendency to a reduction in the inulin clearance which fact was also observed in rats at plasma sodium concentrations exceeding 176 meq/l as reported by Giebisch Klose and Windhager (1964) on the basis of a similar investigation According to *possibility I* the initial increase in filtration rate and sodium reabsorption rate is due to an increased transport capacity (rate of micropinocytosis) At a certain point the latter has reached a maximum (zero inhibition) With increasing plasma concentrations the rate of sodium (and volume) reabsorption remains constant (saturation) all other factors being equal The extra amount of sodium filtered is then excreted With further increments of the luminal sodium concentration the rate of sodium reabsorption decreased corresponding to a decreased micropinocytotic activity (reduced capacity) In the case considered (I) also the volume reabsorbed per min would be reduced It appears actually from the data given by Kruhoffer that the volume of fluid reabsorbed per min was reduced at the very high plasma sodium concentrations It is therefore necessary to postulate that the high sodium concentration (or osmolality) has an inhibitory effect on the luminal activity of micropinocytosis

If interpreted according to *possibility II* the initial rise in rates of filtration and sodium reabsorption is also due to an increased capacity of volume transfer which proceeds until a certain maximum is reached At further increasing luminal sodium concentration a constant rate of sodium reabsorption would indicate that the rate of micropinocytotic channel and vesicle formation (capacity) is reduced along with increased concentrations in the ingested fluid Also in this case the elevated sodium concentration must be assumed to have an inhibitory effect on the luminal process of volume transfer Thus an apparent saturation or sodium Tm is reached at this state of function further elevations of the sodium concentration and inhibition of the micro

pinocytotic' activity may be followed by a decreased reabsorption rate of sodium, giving the appearance of a reduced sodium Tm ('substrate inhibition) As far as I know no data are available which permit a clear distinction between these two possibilities of interpretation

The most reasonable conclusion to be drawn from the above discussion seems to be that *the data forming the basis of the present hypothesis of the mechanism of transcellular transport in the mammalian proximal tubules makes it permissible that the proximal reabsorption rate of salt and water be characterized as Tm limited*

c) *The rate limiting step of the mammalian proximal tubular reabsorption of glucose*

The question now arises to which extent the characteristics of glucose reabsorption in mammalian nephrons may be comprehended and adapted to the above hypothesis The lack of glucose in the final urine at normal plasma concentrations and its presence in the glomerular filtrate in the same concentrations as in plasma clearly indicates that glucose is actively reabsorbed against a concentration gradient It is indicated also in clearance studies (cf p 15) that at increasing plasma concentrations a certain glucose concentration level may be reached above which the rate of glucose reabsorption remains unchanged independent of the filtered load i e that a Tm_G has been reached Furthermore it has been established beyond doubt in several clearance investigations in mammals (e g Kruhoffer 1950) that the glucose Tm remains uninfluenced by induced variations in the rate of filtrate formation above a certain level (10 ml/min in rabbits) and thus in the rate of reabsorption of salt and water In this case salt and water serve as substances for reference Apparently no special problems of interpretation have been attached previously to this fact Above the glucose Tm level a hypothetical carrier was supposed to be saturated In the view of the above hypothesis it may seem as a paradox that the understanding of the Tm limitation of glucose transfer rate represents a problem because according to this hypothesis a Tm limitation of the previous substances for reference (salt and water) may be described as a representative of luminal micropinocytotic activity Thus if the rate of salt and water reabsorption is limited by the micropinocytotic activity under physiological conditions it may apparently be difficult to understand that the rate of net transfer of glucose remains uninfluenced by the rate of micropinocytosis Theoretically, however the difficulties might appear to be less severe The explanation is merely that different steps in the transfer process are rate limiting to salt and glucose

reabsorption the transfer step across the peritubular membrane being rate limiting to glucose reabsorption No data are available by which it may be disclosed or suggested whether or not the limiting step to proximal net transfer of glucose actually is located to the basal (or peritubular) side of the cell a more detailed discussion on the glucose transport in mammalian nephrons must be postponed until more direct evidence has been obtained together with a detailed physico-chemical characterization of the transcellular transfer mechanism(s) involved

The data reviewed in this chapter which have thrown considerable doubt on some of the previously and generally accepted concepts and paved the way for an alternative hypothesis thus challenge future research work on the mechanism(s) of transcellular transport to provide new approaches including biochemical as well as cytophysiological investigations

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about 10 sec and the luminal diameter on an average 31 μ corresponding to a net flux of water of 5.5 × 10^{-4} mm^3 mm sec^{-1} From the flux of water the reduction of flow rate in the lumen at various distances (s) from the glomerulus was calculated from the equation

$$\frac{V}{V_0} = 1 - \frac{2\pi r Ø_w}{V_0} s$$

in which V_0 denotes the flow rate at a distance (s) = 0 from the glomerulus (= rate of filtration from a single nephron), V the flow rate at a distance of (s) and (r) the luminal radius The equation will be seen to imply that the flux term is constant at various distances from the glomerulus in agreement with Gertz observation that the flux did not vary by more than 10 per cent along the accessible part (60–70 per cent) of the proximal segment Inserting in the equation the obtained value of $Ø_w$ (measured at a radius of 15.5 μ) a radius of 10 μ and a value of $V_0 = 4 \times 10^{-4}$ mm^3/sec (calculated by Gottschalk and Mylle (1956) in rats under similar experimental conditions) it was found that the relative fluid volume reduction at a certain distance from the glomerulus agreed fairly well with the one calculated from known data of the tubular fluid concentration index (F/P) for inulin along the proximal tubules suggesting indirectly that the flux per unit surface area remains constant during reductions of the luminal diameter

According to Gertz an automatically adjusted glomerulo tubular balance or load dependent proximal reabsorption rate would now be explained if any reduction in the glomerular filtration rate (or load) were to result in a reduced internal diameter and thereby in a reduced surface area At a constant transcellular net flux per unit area the transfer rate would decrease pari passu with the filtered load This hypothesis is clearly incompatible with some direct observations and physical facts First of all it is in striking contrast to the author's own direct observation that the flux is constant along the accessible part of the proximal convoluted segment in which the flow rate (or load) is rapidly decreasing (about 60–75 per cent reabsorbed at the end of that part) indicating directly that the net flux of water and sodium is independent of the load (i e has the character of a Tm limited process above the saturation level) Furthermore it has been shown (Leyssac 1964 a) that the proximal internal diameters of the major part of the nephron population is uninfluenced by the rate of filtration within a great range of variation Finally changes in the luminal diameter should by no means change the enormous surface area of the luminal brush border of the proximal tubules The relevance of this physical fact was rejected by Gertz because it would imply according to the general concept that the proximal tubular lumina

would be emptied at reductions of the filtration rate within the physiological range of variation (in contrast to the observed facts) if the flux were constant per unit area It was not taken into consideration that changes in the rate of filtration cannot occur physiologically without primary changes in the transcellular flux as might have been inferred from his direct evidence of a proximal salt Tm

b) *Kelman s hypothesis*

Kelman (1962) inferred from a theoretical treatment of micropuncture data a formal analogy between proximal reabsorption of sodium and water and the reaction velocity (or transfer rate) of a tubular catalytic flow reactor, in which the transfer rate is proportional to the fluid flow through the tubular lumen The mathematical formalism describing the transfer kinetics in the proximal tubules in accordance with the model was based on the indication from the data provided by Walker et al (1941) viz that the fraction of the glomerular filtrate reabsorbed up to a given point in the proximal tubules is independent of the filtration rate as substantiated recently by similar direct experimental results (mentioned in Ch I B c) Further it was assumed that the proximal tubule is functionally uniform from the glomerular to the distal end (i e pars convoluta and pars recta) althogether implying that the volume of flow per unit of time past a given point in the proximal tubule decreases exponentially as a function of distance from the glomerulus However, the entire deduction leading to the final conclusion that proximal reabsorption rate is linearly dependent on the filtration rate, and that reabsorption rate 1) at any given distance from the glomerulus is reduced in proportion to the flow rate is based on the implicit assumption ,the relevance of which is not even discussed that the glomerular filtration rate is the independent variable Certain results were deduced from the mathematical development of the model which does not include any sugge tions of the possible mechanisms of a flow *dependent reaction velocity in nephrons These results apparently* were not inconsistent with the experimental micropuncture data taking data of fluid to plasma ratios (F/P) of inulin from the early part of the distal tubule as representative of the fluid at the end of pars recta (Kelman 1962, fig 2) But it is highly questionable as also mentioned by Kelman whether the assumption is correct that pars recta is functionally equal to the convoluted part, since electron microscopic examinations (see Rhodin 1958) have revealed a decrease in microvilli and mitochondria density in the distal parts of the proximal tubules suggesting a decreased reabsorptive capacity in this segment of the tubules Therefore it would be a more convincing support to the Kel

man hypothesis, if data obtained exclusively from the proximal convoluted segment were consistent with the model Such data were recently reported by Lassister Mylle and Gottschalk (1964) and Giebisch, Klose and Windhager (1964) who used saline diuretic rats The F/P inulin obtained in proximal tubules at various distances from the glomerulus were interpreted as being consistent with the Kelman model This model requires, of course that a plot of the inulin ratios versus distance from the glomerulus in a semilogarithmic system should yield a rectilinear curve starting at (1 0) In both reports the data might be fitted to a straight line, but this line clearly intercepts with the ordinate (log scale of F/P inulin) at a point significantly lower than unity This fact however is not easily seen in the figure given by Giebisch et al (1964, fig 5), which may be misleading to the reader, since the ordinate value (1 –) is separated from the abscissa value (–,0) in such a way that the ordinate intercepts with the abscissa value (–,0) at a value far less than unity Thus the directly obtained data are seen to be incompatible with Kelman's model as were also the ones obtained by Gertz (cf section a) Whether the data might fit the theoretical curve, calculated on the assumption of a constant transfer rate along this segment, was not discussed However, a redrawing of the curve on the basis of their data makes it closely adaptable to such theoretical curve Furthermore in the same paper Giebisch et al (1964 fig 1) showed a linear relationship between the percentile proximal fluid reabsorption and the distance from the glomerulus in isotonic and hypertonic NaCl loaded animals, in which the excretion fractions of sodium were less than 12 per cent of the filtered load This demonstration which is in conformity with previously reported results from the same laboratory (Windhager and Giebisch 1961 b fig 3) and conclusions drawn by Walker et al (1941) from their data, indicates a uniform reabsorption rate along the convoluted part of the proximal segment

These data obtained by direct methods are *all inconsistent with the idea of a decreasing reabsorption rate along the proximal convoluted segment* but greatly support the alternative – that the proximal reabsorption rate is independent of the load presented

c) *The general contemporary concept*

Generally the concept of gradient limitation of the proximal tubular reabsorption rates is still accepted in a slightly modified version In his recent monograph, Pitts (1963) uses the term gradient time limitation implying that rates of reabsorption of salt in the proximal tubules are determined by the gradients which can be established and by the time of contact of tubular

fluid with the tubular epithelium The gradient is postulated to be established in the lower part (pars recta) of the proximal tubule (inaccessible to direct examination) and is of course purely speculative At all events it seems to be a singularly unhelpful hypothesis, as it is quite safe to state that 65–70 per cent of the filtered volume has been reabsorbed at this lower part of the proximal segment Thus any possible gradient for sodium here cannot possibly explain the fact that the sodium (or salt) and water reabsorption rates and glomerular filtration rate vary in almost direct proportion as apparent if seen from a purely hydrodynamic point of view (cf Ch I B d p p 23–24) The argument most often advanced against Tm limitation of the proximal reabsorption rate of salt is the apparent fact that doubling the rate of filtration (e g in a dog fed with pure meat) would deliver to the tubules per min about twice the amount of salt they were able to reabsorb if the rate of reabsorption were Tm limited and plasma sodium concentration were above the renal plasma threshold The rate of salt excretion therefore should increase in proportion to the rate of filtration which is in contrast to the observed events The argument is evidently fallacious because if the rate of proximal fluid reabsorption were Tm limited the effective filtration pressure (and glomerular filtration rate) would never increase significantly under physiological conditions until the Tm of salt and water (or volume Tm) had changed

B INVESTIGATIONS BY INDIRECT AND DIRECT METHODS, BY WHICH TO ELUCIDATE THE PROBLEM

a) *Indirect methods*

A variety of experimental procedures have been applied in clearance studies *in attempts to investigate whether changes in the filtered load of salt* is automatically followed by similar changes in the rate of reabsorption of the major fraction of the filtered load Three main types of experiments may be distinguished and shall be discussed in brief

It has been shown repeatedly in dogs (e g Selkurt and Post 1950) and in rabbits (Kruhoffer 1950) that elevation of plasma sodium concentration (and, thus in filtered load) by hypertonic sodium chloride infusion may give rise to an initial increase *in glomerular filtration rate and rate of reabsorption of* salt By further elevation of the plasma sodium concentration a level may be reached at which sodium reabsorption rate remains constant or even decreases with inceasing sodium concentration (above 165–170 meq/l) at a constant filtration rate This type of experiment is of course extremely complex and

difficult to interpret, because relatively long periods of time (more than 10–15 min) elapsed between the induced change in load and the collection of urine for clearance determination Secondary events, as nervous reflex mechanisms changing the renal blood flow and glomerular capillary hydrostatic pressure induced by volume change of the extracellular space changes in the aldo sterone secretion changes in the oncotic pressure etc may complicate a quantitative evaluation of the effect induced by the change in the sodium concentration as the only variable, as emphasized also by Kruhøffer (1960) Realizing this fact Bojesen (1954 a and b) had previously stressed the im portance of a study of the immediate effects of changes in the filtered load of salt and water rather than the steady states and drawn the full consequences thereof in his own experiments (cf Ch I p 24, and below) If anything the observation mentioned above might suggest that the rate of reabsorption of salt, at least at high sodium concentrations in the filtrate does not invariably change in parallel with the filtered load A similar suggestion might be inferred from experiments in which hypertonic saline has been infused into one renal artery, the contralateral kidney serving as a simultaneous control thereby excluding some of the possible secondary effects In this way e g Selkurt (1954) showed by step wise increasing the sodium concentration in the infused solution that a marked increase in sodium excretion rate electively could be obtained without measurable changes in the rate of filtra tion on the side of infusion Simultaneous changes in the urine volume follow ing these changes in the load however make the exact estimation of filtration rates highly difficult, since corrections for dead space errors were not carried out which to some extent obscured the significance of the observation Apparently diametrically opposed results obtained in a series of clearance experiments in dogs, were reported by Kamm and Levinsky (1964) They calculated the rates of sodium reabsorption at different inulin clearances which were varied over a large range partly by pre loading with isotonic saline infusions and/or by desoxycorticosterone (and vasopressin) treatment partly by or combined with graded aortic constriction above the renal arteries After a series of control collections the plasma sodium concentration was elevated by hypertonic saline infusion and the rates of reabsorption were determined at the various rates of filtration brought about either by partial closure or by a reopening of the aortic clamp Now the rates of reabsorption at equal rates of filtration could be compared at high (>170 meq/l) and low (<160 meq/l) plasma sodium concentrations over a large range of variation in inulin clearances They demonstrated that rates of sodium re absorption at any glomerular filtration rate thus effectuated were significantly higher at the high sodium concentration level than at the low level Further

more the amount of sodium reabsorbed per 100 ml of filtrate increased with increasing plasma sodium concentration in the range 140 to about 190 meq/l Since no such difference between animals with and without desoxy corticosterone treatment was observed, secondary changes in the adrenal cortical activity could be ruled out as a reason for this apparent paradoxical observation The somewhat more complicated procedure (aortic constriction) a demonstration of a decrease in the plasma protein concentration on loading and the fact that collections were not made until at least 45 min after start of the hypertonic loadings involves that the interpretation of the increase in the reabsorption rate of sodium at high plasma sodium levels become rather difficult in these experiments It was suggested that the sodium reabsorption might have changed merely as a direct effect of the increased sodium load This suggestion was by no means established by their data Assuming that the proximal tubular reabsorption of salt has the character of a Tm process under intrarenal hormonal control the observation of the relatively increased sodium reabsorption rate after hypertonic loading the glomerular filtration rate being reduced by aortic constriction to any particular value identical to a value in a pre loading period, might suggest merely that a normal balance between glomerular propulsive pressure and the controlled Tm is disturbed under these conditions Any explanation of the seemingly opposed results obtained after hypertonic saline loadings cannot be given until the mechanism(s) by which the hormone release is effectuated have been outlined in greater detail the results might even eventually contribute to a future understanding of such mechanisms

The interpretation of experiments on mechanically changed diuresis may be less complex Shipley and Study (1951) studied the effect of an acute elevation of perfusion pressure in the renal artery of a semi isolated kidney in anaesthetized dog By means of a pumping device blood drawn from a carotid artery of the same dog could be pumped into the renal artery at any desired pressure They showed that increasing pressure within the range of 80 to 180 mm Hg (the autoregulated interval of pressure) was followed by marked diuresis and saluresis without any appreciable increase in the glomerular filtration rate or the renal plasma flow Conversely in acute experiments minor reductions in the renal arterial pressure, induced by partial clamping of the renal artery may reduce significantly the rate of salt excretion on that side without any measurable change in the filtration rate as demonstrated by Blake et al (1950) and Selkurt (1951 a) this fact could also be demonstrated over prolonged periods of time thus excluding dead space errors as an explanation (Mueller et al 1951) A reasonable interpretation of the results obtained from this type of experiments would be that induced changes

in the renal arterial pressure have changed the glomerular propulsive pressure to some extent in the same direction without concomitant, parallel changes in the proximal rate of reabsorption Hence similar changes in the proximal intratubular pressure have occurred which explain satisfactorily the observed changes in the rate of salt excretion as well as the unchanged rates of filtration (unchanged effective filtration pressures) This was actually how Selkurt interpreted these results (Selkurt 1951 b), viz that the experimental results suggested a Tm_{Na} As parallel changes in the glomerular filtration rate and the sodium reabsorption rate were seen to occur, however at major reductions in the renal arterial pressure and since it could not be excluded in the former experiments that changes in the salt excretion might have been due to changes in the distal reabsorption rate the idea of a proximal salt Tm could not be considered established and was given up

The third type of experiments are those in which the glomerular propulsive pressure is acutely changed by rapid infusions of large amounts of saline solutions containing either high concentrations of proteins or no colloids at all (dilution diuresis) Rapid and significant changes in the oncotic pressure may be induced in this way but because of instantaneous and considerable changes in the urine flow the sequence of events and rapid changes in glomerular filtration rate cannot possibly be detected and quantitatively evaluated unless careful corrections for dead space errors are undertaken To the author's knowlegde such corrections have not been applied to this type of experiments except by Bojesen (1954 b) (referred to in Ch I B d p 24) for which reason his experiments exclusively shall be commented here This author showed that an acute lowering of the oncotic pressure even by 8 mm Hg was followed by an instantaneous rise in the urine output and salt excretion rate the glomerular filtration rate the rate of reabsorption and the renal plasma flow remained unaffected until about 10–15 min after the induced change in the glomerular propulsive pressure Within this time interval a direct proportionality was observed between the changes in plasma oncotic pressure over a range of about 8 mm Hg and the changes in the rate of water and salt excretion (Bojesen 1954 b fig 2) Furthermore it was demonstrated that the composition of the urine excreted in excess after the oncotic pressure had been lowered corresponds fairly well to that of an ultrafiltrate as regards chloride and total cations Therefore it was concluded in accordance with the interpretation of results from mechanically evoked diureses mentioned above that salt is reabsorbed as a Tm substance probably throughout the entire nephron Consequently this indirect evidence would infer as emphasized by Bojesen and also in the present review (Chapter I) that the rate of filtration is determined by the rate of reabsorption in the proximal

tubules i e by the actual value of the Tm at that very moment Since the inulin clearance and renal plasma flow had increased 10–15 min after the infusions (II phase) whether or not the solutions contained proteins, Bojesen suggested that some humoral factor(s) presented to the *cells* might have changed the reabsorptive capacity The nature of such substance(s) remained undetermined nor could the reason be established of the prolonged but moderately increased salt excretion rate observed 45–60 min after such volume expansion ("III phase) The only relevant argument against Bojesen's conclusions seems to be that it could not be excluded here that the process of reabsorption might have been inhibited in some way (e g by the change evoked in the oncotic pressure or in the intratubular hydrostatic pressure proper) to exactly the same extent as the reabsorption might have been increased by the elevation in the filtered load Obviously this possibility is highly speculative and would seem less likely

Altogether the possibility of an existance of a rate limiting proximal salt Tm is not precluded in any of the results obtained in these clearance studies If anything they might be suggestive of such concept, or as seen from Bojesen's results only, provide rather strong evidence hereof

In order to understand the physiology and pathology of the kidney it is of obvious and major importance to know whether the proximal tubular salt transfer is Tm limited or load dependent hence it is rather amazing that later investigators apparently have neglected to take into consideration this problem even the evidence presented by Bojesen has been dismissed and it has been declared that the validity of his dead space error corrections remains to be confirmed (e g Wesson 1957, p 320), although the same author aknowledges the value of Bojesen s method by which it has become possible to measure clearances of inulin when urine flows is variable (Wesson 1957 foot note p 282) If, actually later investigators had accepted the question as a problem of importance all efforts would primarily have been focussed on the confirmation or disapproval of the validity of Bojesen s method and results or using the direct methods might have attacked the problem as such For several years such efforts were not mobilized in the field of renal physiology and gradually this problem has vaned off Most authors interpret without further ado their results according to the theories advanced by Smith while Kruhoffer (1960) apparently has accepted the existence of a rate limiting proximal Tm for salt and water In his review Kruhoffer pays the major attention to hydrodynamic considerations in an interpretation of the results (viz possible changes in the glomerular propulsive pressure and resistance to fluid flow along the tubular lumina) This point of view seems to be of more relevance when the *extent to which changes in the proximal reabsorp*

tion rate as an isolated event, may change the intratubular pressure and the glomerular filtration rate without disturbing the sodium excretion and when the consequences of isolated changes in the glomerular propulsive pressure have to be evaluated Since these questions are of secondary importance only to the crucial point whether or not the proximal reabsorption rate is load dependent being relevant only in the case of load independency major attention has been paid to this latter problem in the present study Further hydrodynamic considerations on the pressure decline across the glomerular membrane are apparently of minor importance when a proximal salt Tm or volume Tm is a variable parameter and physiological variations of the Tm are part of a mechanism controlling the proximal intratubular pressure

b) *Direct methods*

An approach to the problem by direct methods permitting an estimate of the tubular function independent of the simultaneous rate of filtration might be possible by the micropuncture technique in its advanced technical perfection It is technically possible by the microperfusion device, described by Sonnenberg and Deetjen (1964) to perfuse at any desired, but constant perfusion rate within the range of 10– 35×10^{-6} ml/min (normal flow rates in proximal tubules of the intact mammalian kidney) into a proximal tubular segment, separated from the glomerulus by an intraluminally injected column of oil and to collect samples for ultra microanalysis of the perfusion fluid at a more distal site By this technique the rate of reabsorption may be determined at different tubular loads independent of the overall functional state of the kidney and possibly without significant interference with an intrarenal regulation mechanism(s) if any This recently developed method has not yet been applied to investigations of this type Some fundamental informations, however, are obtainable from the conventional micropuncture technique by analyses of samples collected at various later determined distances from the glomerulus, as already mentioned (section A a and b in the present chapter) The value of this conventional technique is limited particularly as regards the problem here discussed, whether the observed *rapid changes* in the state of tubular function may be due primarily to changes in glomerular vascular factors or to simultaneously occurring changes in the renal blood flow cannot be decided on the basis of results thus obtained

It was of great interest when Hanssen (1960) provided evidence that fluid in the proximal tubular lumina at the time of interruption of the renal circulation (and filtration) is continously reabsorbed into the interstitial space

until the convoluted part (4/5 of the proximal tubule) is emptied Ferrocyanide, known to be excreted by filtration only was injected intravenously into mice in vivo the left exteriorized kidney was subsequently frozen at the moment of sacrifice 5 sec after the injection, the intraluminal distribution of ferrocyanide, precipitated as Prussian blue was examined in nephrons isolated by microdissection The distribution of ferrocyanide in tubules of the left snap frozen kidney was compared with the distribution in the tubules of the right kidney, frozen at different, stated time intervals (20–300 sec) after the animal was sacrificed It was observed that ferrocyanide in the upper 4/5 of the proximal tubules was not displaced to any significant degree and did not pass the bend of the loop of Henle A displacement in a proximal direction was never observed Furthermore the intertubular space in kidneys, the renal pedicles of which had been tied before freezing were seen to be very wide at the time when the proximal tubules were occluded in contrast to a narrow intertubular space seen in kidneys frozen at the moment of interruption of the blood circulation which observation has been confirmed by the present author's experiments (cf Leyssac 1965 a figs 4 and 5) Consequently a fair agreement could be anticipated between the tubular fluid content and the amount of draining fluid collected from the renal vein after removal of the kidney, because some intertubular space still remains in the cortex of a drained kidney Such fair agreement was actually observed Swann (1960) using rats found a value of 0 230 ml/g of moist tissue drained from the kidney this value was confirmed by Little and Robinson (1963) who found that 0 217 ml/g of moist tissue actually was drained, and 0 248 ml/g of moist tissue was lost as deduced from analyses of tissue frozen in situ and after exsanguination These latter investigators demonstrated also that the composition of the extra fluid lost from the kidney upon exsanguination resembled extracellular fluid (139 meq Na^+ 127 Cl^- per kg of water, but no considerable amounts of K^+) These values agree with the volume of proximal tubular fluid in rats viz 0 18–0 19 ml per kidney in animals with a kidney weight of an average of 0 87 g (equal to 0 21–0 22 ml/g of tissue) calculated by Leyssac (1963) on the basis of the known number of nephrons and dimensions of the lumen It would be justified to state on the basis of these arguments that the proximal luminal occlusion observed in the early period following interruption of the renal circulation is due to a continued reabsorption Consequently the rate of disappearance of the lumina measureable in situ in the rat by in vivo microscopy, might serve as a measure of the rate of proximal reabsorption (i e of the volume of proximal fluid reabsorbed per unit of time and unit of tubular length) Provided that the composition of the tubular fluid and the volume of fluid per unit of tubular length of the

proximal segment (i e the internal diameter) is kept reasonably constant from one experiment to the other, a difference in the recorded time interval (denoted as occlusion time') from interruption of the circulation until the major part of the proximal lumina has disappeared would indicate directly a difference in the rate of transfer of salt and water By this method described by Leyssac (1963), it was observed that the lumina were occluded on an average 18 5 sec after interruption of the renal circulation at inulin clearances of the lower range (about 1 ml/min/g KW) which was the state of function most frequently observed in the laparotomized and moderately saline loaded animals used here (body weight av 250 g) (whole material Leyssac 1965 b) The in vivo observation was confirmed by the histological findings of almost completely occluded proximal tubules in kidneys frozen 18 sec after removal from rats under similar experimental condition (Leyssac 1965 a fig 5 and Steinhausen et al 1963) the latter authors microphotographed at rapid successsive exposures the kidney surface in antidiuretic rats after interruption of the renal circulation In order to make the lumina contrast the bright cellular lining and brush border Lissamin green was administered intravenously prior to the arterial clamping By this technique Steinhausen showed that the proximal lumina in rats were occluded within 20 sec i e the occlusion time most frequently seen in antidiuretic and moderately diuretic rats The fairly good agreement between reabsorption rates of salt and water in rats evaluated by measurements of the occlusion time and by other methods would establish that the occlusion time method may be used for estimations of the proximal reabsorption rate It was found that the occlusion time to be expected if calculated from the immediately preceding clearance of inulin (and on the assumption that the proximal reabsorption continues at an unchanged rate until the lumina were emptied) was in rather close agreement with the rate actually measured over the entire range of spontaneous variation (e g av 16 5 sec calculated and av 18 5 sec observed at a clearance of 1 0 ml/min/g KW, Leyssac 1963 1965 b) Agreement between these two independent methods by which to estimate the rate of proximal reabsorption in the same animal and at the same time was found to be equally satisfactory in young animals (body weight 100 g) in which dimensions of the nephrons are much smaller than in adult rats (Leyssac 1965 c) A similarly fair correspondence is found even if related to reabsorption rates in the proximal tubules of rat kidneys, as reported by other investigators using other direct methods With an internal radius of 12 5 μ (a value between the ones obtained histologically in snap frozen kidneys by the author and the ones obtained in vivo by Steinhausen et al (1963) (cf Leyssac 1965 c)) together with a plasma sodium concentration of 150 meq/l a 1 μ thick cross section of a proximal

tubule will contain 7 35$\times$10^{-8} μeq Na in 490 μ^3 of luminal fluid With an occlusion time of 18 5 sec and an external radius of 17 5 μ (deduced from the observation of a transsectioned cellular area to luminal area ratio about unity (Leyssac 1965 c)), 3 6$\times$10^{-} μeq/mm sec are found to be transported across the peritubular membrane or 5$\times$10^{-} μeq/mm^2 sec transported across the luminal membrane (considering the lumen as a cylinder with a radius of 12 5 μ) This value may be related to the value of 3 0 – and 3 4$\times$10^{-5} μeq/mm sec reported by Windhager and Giesbisch (1961 a) estimated on the basis of data obtained by the stop flow microperfusion technique in single proximal tubules of rats and by short circuit current measurements, respectively, and to the value of 8$\times$10^{-5} μeq/mm sec obtained by Gertz (1963) using another modification of the micropuncture technique, referred to previously (p 52–53)

The close agreement between the observed occlusion time and the one calculated from the immediately preceding clearance of inulin on the assumption of a constant and unchanged reabsorption rate after interruption of the renal circulation and filtration indicates that the reabsorption actually does continue in the proximal convoluted segment at a constant rate until no filtrate is left within the lumen, a direct observation permitting the conclusion to be drawn that the rate of reabsorption is independent of the filtered load per se (Leyssac 1963)

Based on the same assumption the relationship between reciprocal occlusion time and inulin clearance per g of kidney weight (KW) should remain unchanged with uniform fractional reabsorption proximally and uniform total fluid volume of the proximal convoluted segment per g KW ($\frac{V}{KW}$) Data obtained in experiments using small rats (100 g body weight) (Leyssac 1965 c) indicated that this relationship did not differ significantly from the ones seen in adult rats Accepting that the fractional proximal reabsorption of filtrate i about the same in young and adult animals, $\frac{V}{KW}$ should be equal in these two groups of rats always provided that the basic assumption is valid It was observed that the ratio cross sectioned proximal luminal area to cellular area was approximately the same in the two groups and that the luminal volume per unit of length was proportional to the kidney weight $\frac{V}{KW}$ therefore would be equal in the two groups if the length of the proximal tubules is equal in 100 g rats and 250 g rats This is a reasonable assumption as it has been shown by Fetterman et al (1964) by microdissection that the final step in the development of the proximal convoluted tubules in man in the age group 15–21

years is an increase in the volume without further increment in the segmental length In children at the age of 15 years the proximal volume was found to be at an average of 0 0423 mm³ as opposed to an average volume of 0 0875 mm³ in adults In accordance with this the kidney weight was twice as high in adult rats as in rats of 100 g Thus the data obtained in small rats supports the conclusion that the rate of reabsorption continues at an unchanged rate during the luminal occlusion period after cessation of the glomerular filtration independent of the load

Thirdly the fact that the great majority of the proximal convoluted tubules in a field of vision complete the luminal occlusion within plus or minus 2 sec may possibly be taken as additional evidence in the discussion of a load dependency of the proximal fluid reabsorption and may further serve as basis for a brief comment on the limitations of the occlusion time method The fairly well defined end point of luminal occlusion of the visible two thirds of the proximal tubules might apparently suggest that fluid reabsorption is completed almost simultaneously along this segment since the luminal fluid is not displaced to any significant degree either distally or proximally according to arguments discussed above (p 62) Since the flow rate of luminal fluid (and thus the load) is rapidly decreasing almost exponentially along this tubular segment because of reabsorption an equal rate of fluid reabsorption here would indicate that the proximal reabsorption rate is independent of the load A quantitative estimation however will disclose that the measured occlusion time *alone* does not permit this conclusion Thus it might be postulated that the distribution of proximal internal diameters in control rats (cf Leyssac 1964 a fig 7) represents the distribution of diameters along equally sized but conical segments in such a way that the major group of measured diameters (about 27 μ) represents the diameter of the middle half of the convolution the group with larger diameters (about 32 μ) represents the diameter of the early one fourth of the segment, and the group with small diameters (about 22 μ) the distal fourth of the convoluted part Moreover if it is postulated that an occlusion time about 18 sec (16–20 sec) represents a mean of different occlusion times along the segment with the early part occluding first (in 16 sec) and the distal one fourth last (in 20 sec), *it will appear that the rate of fluid reabsorption decreases almost exponentially* (from about $3\,2 \times 10^{-4}$ mm³ mm⁻ sec⁻¹ to $1\,75 \times 10^{-4}$ mm³ mm⁻ sec⁻¹) and parallel to the rate of fluid flow along this segment It therefore must be concluded that measurements of the occlusion time are too inaccurate per se to exclude the possibility of an exponential decrease in the reabsorption rate along the proximal convoluted segment Steinhausen et al (1963) observed however in photographic serie-exposures that the luminal occlusion

proceded from the distal end of the proximal convolution towards the glomerulus If this fact is included in the above considerations (always maintaining the postulate of the conical shape of the segment) it would mean that the distal parts occlude in 16 sec and the early parts in 20 sec Then the rate of reabsorption will appear to remain almost unchanged along the convoluted segment (2.5×10^{-4} mm^3 mm^{-2} sec^{-1} in the early part as opposed to 2.2×10^{-4} mm^3 mm^{-2} sec^{-1} in the distal part) Finally it seems more reasonable if the distribution of the internal diameters is considered to be representative mainly of diameters in nephrons of unequal sizes It may then be concluded that the fairly well defined end point of the luminal occlusion in the major part of the visible convolutions actually excludes the possibility of decreasing reabsorption rate along the proximal convoluted segment, the additional information provided by Steinhausen s observation being taken into account In other words the well defined end point of occlusion serves as further direct evidence of the feature that the proximal reabsorption rate is independent of the load Finally it should be borne in mind that data obtained by two different micropuncture techniques — Gertz flux measurements and determinations of F/P inulin ratios along the proximal tubule —, although differently interpreted by the authors (cf section A a and b of this Ch) clearly had indicated that the proximal reabsorption rate along this segment is independent of the load, in accordance with the latter conclusion drawn from the occlusion time method

It should be emphasized however that even if direct measurements had shown that the proximal reab orption rate decreases exponentially along the segment as opposed to the demonstrated fact, such evidence need not at all have invalidated the concept of Tm limitation of the rate of fluid transfer across the tubular cell The demonstrated close agreement between the occlu sion time calculated on the preceding inulin clearance and the one directly observed (cf p 63) representing the occlusion time of a tubule section with the mean diameter indicate beyond doubt that the local fluid reabsorption (across the single cell) continues at an unchanged rate during the occlusion period independent of the load Naturally this fact has not excluded a priori that different (decreasing) transport capacities (or Tm levels) might have existed along the proximal convolution However observations by three in dependent methods (1) by the flux measurements, 2) the F/P inulin ratios, and 3) the well defined end point of occlusion) that the rate of fluid re absorption along the proximal convoluted segment is independent of the load presented may be characterized as additional evidence of the concept of Tm limitation of this rate

The existence of spontaneous variations in this rate indicates that the

Tm is variable, at least within this range Moreover, below the spontaneous minimum value the rate of reabsorption is not changed significantly (Leyssac 1964 a fig 2), as estimated from the occlusion time, by reductions in the rate of filtration even not if the inulin clearance is reduced far below the spontaneous range of variation of this parameter, e g by partial clamping of the renal artery The requirements of uniform composition of the luminal filtrate and identical internal diameters in the major part of the proximal tubules were apparently fulfilled in these experiments It may be questioned whether the skew distribution of internal proximal diameters in these clamped kidneys including many tubules of small calibre (cf Leyssac 1964 a fig 7) may have affected the registered end point of occlusion thus rendering it less well defined This possibility cannot be excluded and probably it may become of significance at the very low clearance values (below 0 5–0 6 ml/min/g KW) thus involving that the occlusion time will be too brief But the method being used as and only permits, a rather rough estimate of the proximal reabsorption rates this objection cannot invalidate the conclusion that the proximal Tm for salt and water has reached a minimal fixed value at rates of filtration below the minimal spontaneous value, depressed (artificially or pathologically) by a lowering of the glomerular propulsive pressure At these low clearance values the proximal salt – or volume – Tm is invariable like the glucose Tm At these *low* rates of filtration right up to the minimum normal rate reductions in the filtration rate (or more correctly in propulsive pressure) should bring about tubular occlusion (cf the argument by Gertz Ch III A p 53–54) Actually it was found that below this rate of filtration the urine flow would decrease in direct proportion to the inulin clearance indicating that a decreasing number of nephrons participate in the urine formation pari passu with decreasing filtration rates Besides occluded proximal tubules were histologically demonstrated in kidneys frozen at this state of function as opposed to the wide open lumina seen in all tubules at higher clearances The demonstration of a proximal luminal occlusion at these low rates of filtration represents a further (fourth) demonstration of the proximal Tm limitation of the fluid reabsorption rate

The conclusion to be drawn is that *investigations by direct methods have definitely shown that under normal conditions the rate of proximal tubular fluid reabsorption is Tm limited in mammalian kidneys*

As the proximal fluid reabsorption has the character of a bulk reabsorption (cf Ch II p 41) and since variations in the reabsorption rate apparently are variations in the transfer rate of a volume of an aqueous solution of electrolytes and urea it follows that reabsorption rates not only of sodium but also of chloride, bicarbonate, potassium and urea apparently are limited

by one and the same step in the transfer process It may surprise that this fact (Tm limitation) has escaped recognition and discussion in most of the previous investigations One explanation may be that the Tm apparently is rapidly variable which has not been considered (at any rate not been expressed) except by Bojesen apparently because the concept of Tm has arisen from and later in general been attached to, a specific method used in a particular experimental situation, by which method such rapidly variable Tm is not demonstrable Bojesen seems to be the only previous investigator who has designed experiments by other methods in order to demonstrate (or disprove) a Tm process of this type This deranged way of thinking has also impeded the interpretation of such micropuncture data which might have provided some evidence it even prevented the authors from analysing the untraditional possibility of a variable Tm on the basis of their own data It seems rather irrelevant to define theoretically any concept on such criteria It is generally agreed that the distal transfer capacity of sodium is Tm limited and that the capacity may be changed by the action of mineralocorticoid hormones within 45 to 60 min Although the conventional clearance method fails to demonstrate changes in the proximal Tm occurring in less than 20–30 min it is by no means justified to infer that the proximal transfer process *on principle* cannot be Tm limited The criterion is not whether the rate of transfer may be changed in 40 20 10 or one min The time interval necessary for a change to occur is solely determinant for the methods by which the Tm character may be demonstrated

As a consequence of the above presented arguments the concept would arise that there is *on principle* no difference between the process of reabsorption of salt (or sodium) in the proximal and distal segment of the mammalian nephron In both segments the process is Tm limited and may be varied rapidly in the proximal segment and more slowly in the distal segment

If this solution of the problem concerned (the problem of load dependency versus Tm limitation of the proximal reabsorption rate of salt and water) is now accepted as the only reasonable conclusion to be drawn from the data presented it may be seen already that it has a great bearing on the interpretation of changes occurring in the rate of glomerular filtration

CHAPTER IV

Consequences of Tm-limitation of proximal reabsorption of salt and water

A THE CONCEPT OF THE GLOMERULAR FILTRATION RATE

It was previously argued (Ch I B d p 23–25) that an increase in the glomerular propulsive pressure would be accompanied by a similar increase in the proximal intratubular pressure unless either the tubular resistance to flow was reduced or the rate of proximal reabsorption was increased This statement was based on observations in rats under normal non diuretic conditions under this condition a pressure difference of 12–13 mm Hg was found between the end of the proximal convoluted segment and the renal pelvis through which distance no more than 20–25 per cent of the filtered fluid volume is propulsed If the resistance across the glomerular membrane were equal to the resistance offered by these distal segments of the nephrons a pressure drop (i e an effective filtration pressure) of at least 50–60 mm Hg would be required to account for the actually filtered volume With a mean oncotic pressure in the glomerular capillaries of about 30 mm Hg the hydrostatic capillary pressure should be at least 80–90 mm Hg which of course is unrealistic No doubt the resistance to flow across the glomerular membrane must be less than the resistance offered by the nephron The actual effective filtration pressures however, remain unknown Pappenheimer (1955) tried to calculate the filtration coefficient (ml filtered per min per mm Hg effective filtration pressure) from observations on the glomerular sieving of myoglobin and ovalbumin in dogs A hypothetical pore radius and the filtration coefficient can be calculated by substituting the known diffusion coefficients the Einstein Stokes radii of the protein molecules and the restriction of filtration of these proteins $(C_{\cdot}/C_1)$ in the sieving equation

$$\frac{C}{C_1} = \frac{1+\frac{D}{Q_f} \times \frac{8\,\eta\,k_f}{r}}{\dfrac{1+2\,4\,a/r}{2\,(1-a/r)^2-(1-a/r)^4}} + \frac{D}{Q_1} \times \frac{8\,\eta\,k_f}{r^2}$$

in which (a) denotes the radius of the protein molecule (r) the pore radius (Qf) the filtration rate (ml/min) (D) the diffusion coefficient (η) the

viscosity, and (Kf) the filtration coefficient By substituting the Einstein Stokes radii a spherical shape of the molecules has to be assumed However, neither myoglobin nor ovalbumin molecules are spherical, nor were corrections for electrical hindrance due to negative charges of the proteins included in Pappenheimer's calculation Thus the pore diameter of 75 Å calculated for dog glomerular membranes is probably an underestimation Moreover, as the pore radius in the fourth power (Poiseuilles law) determines the filtration coefficient, the estimation of the latter is probably also somewhat under estimated and was found to be on an average 3 1 (1 9–4 5) ml per min per mm Hg per 100 g of kidney weight (KW) Based on a filtration rate in dogs of about 6 ml per min per 100 g KW a pressure drop across the glomerular membrane of 20 mm Hg (Δ P) was calculated from

$$\Delta P = \frac{Q_f}{K_f}$$

From similar clearance experiments in dogs using a dextran preparation including molecules of different molecular weights Wallenius (1954) de termined in each sample the distribution of the different molecular weights (compiled in groups with an interval of 4 000) and calculated the renal clearance of each molecular class expressed as a percentage of the simultane ously determined clearance of creatinine (Wallenius 1954 Table 12) By relating the clearance of each group to the representative molecular weight (Fig 20) a curve was constructed which approached zero filtration at a molecular weight of about 50 000, corresponding to an Einstein Stokes radius of about 50 Å The advantage of using dextran is that these molecules are uncharged and no electrical hindrance occurs Hence a pore diameter of about 100 Å might probably be a more correct value for the calculation of the filtration coefficient Using these results Wallenius and Garby (quoted from Kruhøffer 1960 p 264) calculated a pressure drop of only a few mm Hg across the glomerular membrane Wallenius data however, are subject to the objection that the dextran fractions were not quite homogenous although they precipitated as representative of an absolutely destinct fraction Thus the determination of the low concentrations of high molecular weights are questionable and probably underestimated

Of course estimations like those mentioned above should be considered with a *certain reservation* because of the many unestablished *assumptions* underlying the calculation One of the serious objections has been that pores in the basement membrane of capillaries could not be demonstrated by elec tronmicrographs using the conventional techniques although this structure was found to constitute the main filtration barrier to ferritin molecules (mole

cular diameter about 100 Å) in rat glomeruli (Fahrquhart Wissig and Palade 1961) Therefore it may be of interest that Faarup and Christensen (1965) were able to demonstrate a porous structure of the dense layer of the base ment membrane of peritubular capillaries in the medulla of rat kidneys in freeze dried specimens frozen at the moment of removal of the kidney, this procedure might give a more true picture than the one obtained by the conventional technique at which tissues are fixed several minutes after cessa tion of the organ function The parallel pores perpendicular to the membrane surface, had a diameter of 100–300 Å Still it remains to be established however, whether or not this observation might be the result of an artifact due to the applied technique

Were any significance to be attached to these indirect estimations the resistance to fluid flow across the glomerular membrane would apparently be inferior to the resistance offered by the distal tubular segment (from the end of the proximal convolution and throughout) Since the Tm limitation of the proximal reabsorption rate of salt and water is now established the proximal intratubular hydrostatic pressure should vary as a primary event almost parallel with changes in the glomerular propulsive pressure The in direct observation by Bojesen (1954 b) and the direct observation by Gott schalk and Mylle (1956), mentioned previously (Ch I, B d), that such parallel changes do occur immediately after induced changes in the propulsive pressure may be considered a substantial experimental support to the main conclusion drawn from the above calculations *It follows that significant changes in the effective filtration pressure* (or filtration rate) *without violent changes in the excretion rate of salt and water* (due to a reduced resistance to flow) *will not occur until some factor(s) has changed the Tm of the proximal reabsorption Detectable changes in the rate of filtration are there fore reflections of primary changes in the proximal reabsorption rate of salt* and measurements of the inulin clearance should not be considered as any measure of the hydrostatic pressure level in the glomerular capillaries as determined by afferent and/or efferent arteriolar tone (i e of glomerular propulsive pressures) rather such measurements indicate the intrarenal turn over rate*) of salt and water so long as the excreted fraction remains small The realization that turnover rates of salt and water in the range of physiological variation are determined by the rates of proximal reabsorption and that these rates represent different states of a variable Tm process has an important implication to the interpretation of clearance data In this realization is evidently implied that even though the clearance of inulin

*) In this context the term turnover' is defin d without reference to the pool concept

might be determined exactly without any error and variations at all within the collection period it remains theoretically impossible to deduce from measured clearances and excretion rates of water and predominant electro lytes whether changes in the rates of excretion are caused by changes in the glomerular propulsive pressure or by changes in the tubular reabsorption rates The correctness of this statement is apparent from the fact that the rates of excretion are determined not only by the effective filtration pressure and the overall rates of reabsorption but also by the tubular flow resistance and the distribution between proximal and distal reabsorption Because of its importance to the interpretation of clinical and pathophysiological studies in human subjects this statement shall be illustrated in some detail

By way of example a condition of reduced inulin clearance and greatly increased excretion rate of salt may be visualized The overall rate of reabsorp tion would be clearly reduced 1) This condition might be brought about at an unchanged glomerular propulsive pressure and tubular resistance in which case the proximal as well as the distal reabsorption rate are decreased Because of the decreased proximal reabsorption rate the proximal intratubular pressure would be increased (lowering the effective filtration pressure) and the flow rate to the distal segments would increase If the distal reabsorption rate also is assumed to be relatively more reduced than that in the proximal segment the rate of excretion would be increased not only because of the overall re duction in the transfer rate but also on account of the changed distribution between the proximal and distal reabsorption 2) A similar clearance and excretion pattern might be observed in a condition in which the glomerular propulsive pressure is moderately augmented and the proximal and distal reabsorption rates of fluid are decreased relative to the same degree The fraction of the filtered volume excreted would then be somewhat greater than in the former condition and the increased excretion rate of salt would be attribut able partly to the change in the propulsive pressure partly to the change in the tubular function 3) Finally an increased excretion rate of salt in a state of reduced filtration rate might also be obtained in cases in which the propulsive pressure is reduced and the overall reabsorption rate decreased in such a way that the proximal reabsorption rate is greatly inhibited and the distal reabsorption rate is increased In this case it might be assumed that the resistance offered by the loop of Henle is reduced or abolished Then the proximal and distal intratubular pressures might approach each other at a value a little above that normally found in the distal tubules (e g 10 mm Hg) and the effective filtration pressure might still be reduced because of the reduction in the glomerular propulsive pressure The part played by the distal reabsorption in the overall reab orption would be relatively increased and the

increased rate of excretion of salt would be the resultant of a decreased propulsive pressure decreased resistance to flow and a changed distribution between proximal and distal reabsorption

It may also be worth while to consider a condition of a greatly impaired and unchangeable tubular reabsorption throughout the whole nephron At a normal glomerular propulsive pressure (and unchanged resistance) the rate of filtration will be reduced If now the glomerular capillary hydrostatic pressure is increased (e g by afferent arteriolar relaxtion) the rate of excretion will also be increased and the augmentation will correspond to the actual increase in the rate of filtration In such a condition the rate of filtration might reach a normal value at a state of violently increased excretion rates of salt and water which might possibly amount to even more than 50–60 per cent of the filtered volume The intratubular pressures and resistances in the nephron would of course, be highly disturbed in this imaginary case which cannot be compared with normal conditions

Finally it should be mentioned also that since determinations of the turnover rate of salt by the clearance method in its usual performance invariably involves a variability of 5–6 per cent or more between the individual collection periods it may theoretically be observed in human subjects in whom the inulin clearance is unchanged that the rate of excretion of sodium may change within plus or minus 700–800 µeq/min (at a plasma sodium concentration of 140 meq/l) solely as a consequence of changes in the glomerular propulsive pressure i e at an absolutely unchanged rate of sodium reabsorption

In view of the present concept that significant changes in the effective filtration pressure in the individual nephrons (i e changes corresponding to measurable changes in the rate of filtration of the entire kidney if parallel changes occurred in all the nephrons) normally are mediated only by changes in the proximal reabsorption rate of fluid some rigidity of the proximal tubular wall has to be assumed Otherwise moderate changes in the filtered load would be followed only by a corresponding change in the tubular diameter with no appreciable change in the intratubular pressure and moderate changes in the intratubular pressure and thereby in the effective filtration pressure induced by primary changes in the rate of reabsorption could not be established It migh be questioned whether or not the proximal tubules actually do behave normally as more or less rigid tubules Gottschalk and Mylle (1956 1957) showed that the proximal intratubular pressure always was almost identical with the simultaneously measured peritubular capillary pressure in the same kidney in control (i e non diuretic) rats and these pressures increased in parallel within a range of 10 to 50 mm Hg no matter whether this

pressure increase was induced by renal venous compression, ureteral occlusion, or osmotic diuresis This observation might suggest that the tubular wall would give way to an increase in the pressure from the outside as well as from the inside of the tubule until identical pressures were obtained in the interstitial space (capillaries) and the lumina The applied pressure changes, however, exceed by far the physiological interval of pressures and although the results indicate that the suggestion largely is valid for induced, considerable changes in the pressures they fail to give any information whether or not this might also be true within the physiological range of pressure changes because at these low values a scatter of 5–10 mm Hg is seen (By the method used a pressure can be determined with an error of less than one mm Hg) The observation that proximal intratubular pressures within a single normal kidney in a stable state of function may range between 8–10 and 18–20 mm Hg with a mean pressure of about 13–14 mm Hg (Gottschalk and Mylle 1956 and the personal unpublished observation by the present author) actually indicates that pressure differences may exist between single proximal lumina and the surrounding interstitial and intratubular pressures Repeated measurements of the intratubular pressure at the same site of puncture in a proximal convolution showed also that it would either remain constant for some minutes at least or vary over a range of several millimeters of mercury although the experimental conditions remained unchanged Moreover it was demonstrated by registration of the pressure change in proximal tubules and peritubular capillaries following either abrupt clamping of the aorta above the renal arteries or injection of a large dose of a vasoconstrictor substance (angiotensin) that the proximal tubular wall – at least during short term changes in hydrostatic pressures – can resist a moderate positive pressure both from the outside and the inside of the wall (Leyssac 1965 a) By abrupt clamping of the aorta the peritubular capillary pressure dropped to zero almost immediately whereas the proximal intratubular pressure only had reached a value of 6–7 mm Hg in about 3 sec at which time the capillary pressure was zero to decline gradually to zero 12–20 sec after clamping indicating that the tubular wall can resist such positive pressure from the inside for this short time interval Occasionally the peritubular capillary pressure was found to increase to a peak following the injection of angiotensin whereas the proximal intratubular pressure invariably decreased immediately after similar injection, indicating that the tubular wall also can resist a moderate positive pressure from the outside Thus the cellular wall of the proximal tubules seems to be characterized by some rigidity sufficient to resist moderate differences of pressure in the lumen and the interstitium This fact is true at least for short term pressure changes but may possibly be valid also when the changes are

maintained for more prolonged intervals of time (minutes) The physical characteristics of the proximal tubular wall are apparently compatible with the concept that primary changes in the proximal Tm for salt and water may be responsible for the changes observed in the rate of glomerular filtration within a certain range which is not yet quite defined

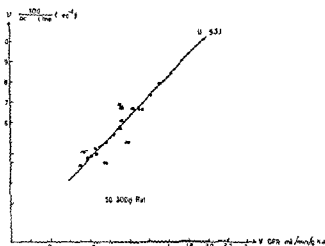


Fig 1 *Reciprocal occlusion time (U) at spontaneously different clearance of inulin (V) in control rats* (Leyssac 1965 b)

Fig 1 demonstrates the direct proportionality between the clearance of inulin and the reciprocal of the occlusion time (representing the rate of proximal reabsorption) in normal rats It is seen that the spontaneous variation in the rate of reabsorption (a variation in the reciprocal occlusion time from 1/11 to 1/25 sec^{-1}) corresponds to a variation in the rate of filtration from about 1 6 ml/min/g KW to about 0 8 ml/min/g KW As it has been shown directly (see Ch III) that the rate of reabsorption is the independent variable (Tm limited) some factor(s) must be looked for in order to understand how these occurring changes in the Tm are effectuated It was suggested by Bojesen (1954 b) that some humoral factor(s) may be influencing the Tm because changes in the reabsorptive capacity were seen to be related to changes in the renal plasma flow Obviously such search would seem meaningless if the reabsorption rate were to change as an automatic consequence of a change in the filtered load

Goormaghtigh (1940) was the first to attribute an endocrine function to the juxtaglomerular apparatus primarily because of the histological appearance

of the granular, epithelioid cells of the afferent arteriole (polkissen) at the very end of that vessel According to Barjas and Latta (1963) and Faarup (1964) the juxtaglomerular apparatus is defined as the afferent and the efferent arterioles containing the epithelioid cells, cells from the adjacent distal tubule (the macula densa), and the Goormaghtigh cells interposed between the macula densa cells the arterioles and the glomerulus In the following discussion the latter definition of the juxtaglomerular apparatus shall be used From serial sections of this anatomical structure from subcapsular and juxta medullary nephrons Barjas and Latta (1963) and Faarup (1964) reconstructed three dimensional models which show that the ascending limb of Henle always approaches the efferent vessel first following it to the vascular pole of the glomerulus here the macula densa cells contact the Goormaghtigh cells and usually the epithelioid cells of the efferent and afferent arterioles The distal tubule (here defined from the macula densa to the collecting duct) will then, after a few convolutions apart from the glomerulus, join and follow for some distance the afferent arteriole before it merges with a distal tubule of another nephron into a collecting duct This peculiar anatomical arrangement characteristic of each mammalian nephron, has naturally puzzled many investigators besides Goormaghtigh who was the first (1940) to suggest on purely morphological evidence that renin – a proteolytic enzyme – is located to the juxtaglomerular cells (epithelioid cells) Later, extensive investigations have firmly established that renin is located and probably formed in the juxtaglomerular apparatus Thus Cook et al (1956) and Bing and Wiberg (1958) showed by direct renin bio assay that the subcapsular aglomerular zone (cortex corticis) did not contain measurable amounts of renin nor did the glomerular capillary tufts removed by microdissection, Cook and Pickering (1959) demonstrated conclusively that only the glomerular fraction contained renin viz by isolating selectively magnetic iron dioxide loaded glomeruli from a suspension of kidney fragments using an electromagnet They also showed that the renin in this fraction was found in the one half of the glomeruli containing the vascular pole Further microdissection studies by Bing and Kazimierczak (1960) confirmed this result but also showed that isolated afferent arterioles contained less than the total juxtaglomerular renin content and that the perivascular cell fraction contained even more renin than did the vessel Well over 50 to 90 per cent of the renin was found actually in the part of the distal tubule containing the macula densa and a smaller fraction less than 50 to 10 per cent was located in the isolated vas afferent which could not be completely separated from the adjacent macular and/or Goormaghtigh cells (Bing and Kazimierczak 1962) These results, indicating directly that the greater part at least of the renin is located in the macula

densa and/or Goormaghtigh cells are apparently contrary to observations by Edelman and Hartroft (1961) who found that fluorescent antibodies to a purified (although not quite pure) renin preparation gave a specific staining of the granular epithelioid cells of the afferent arteriole (JG cells), whereas no staining was seen in the macula cells Furthermore it has been shown repeatedly (e g Tobian 1960) that a correlation is rather fair between the renal renin content and the granularity of the JG cells Kaplan and Friedman (1942) demonstrated the presence of renin in the meso – and metanephros of the hog foetus lacking epithelioid cells, the finding has been confirmed in recent microdissection studies by Bing and Kazimierczak (1964) using kidneys of new born pigs in which renin was found in the subcapsular zone including premature macula cells but devoid of afferent and efferent vessels consequently it can hardly be doubted that renin is located at any rate in the macula densa According to Bing (1964) it is most likely that renin is formed in the macula densa cells from which it passes to the epithelioid cells of the vessels where it is deposited (possibly as or in the granules) subsequently it may either pass into the blood circulation as renin or come into reaction with angiotensinogen (an α_2 globulin) from the plasma In the latter case the product of the enzyme reaction – the decapeptide angiotensin I (Skeggs et al 1957) – might be released directly into the circulation to be immediately converted to the highly potent vasoconstrictive octapeptide angiotensin II (Skeggs Kahn and Shumway 1956)

The location of the vasoactive renin angiotensin system specifically to the characteristic structural arrangement of the juxtaglomerular apparatus together with ample evidence of the fact that the renin content is influenced by the salt balance and the action of mineralocorticoid hormones (cf Ch V B) made it rather suggestive that angiotensin in addition to its renal vasoconstrictive action might also be the humoral substance which is responsible for the changes occurring in the proximal salt and water Tm i e might be the factor foreseen by Bojesen (1954 b 1955 1957)

B THE TUBULAR ACTION OF ANGIOTENSIN

Previous attempts to demonstrate a primary tubular effect of angiotensin were complicated by the invariable and immediate vasoconstrictive effect following the administration of the pressor substance As appears from the foregoing discussion (p 71–73) such effect could not be revealed by clearance studies Hence it can hardly surprise that apparently conflicting conclusions have been drawn when such data obtained in studies on different species of

animals as well as in studies on normal as opposed to diseased human subjects were to be interpreted. Nor could it be expected that such effect could be established by direct methods (micropuncture studies) in experiments in which the renal blood circulation was intact (as tried by Ullrich, personel communication), since effects, if any, will be accompanied by a significant change in the plasma flow and probably also in the glomerular propulsive pressure, obscuring whether a change observed in the tubular function is a primary or secondary event. Another direct approach was tried by Gertz (1962) who microinjected a saline solution containing angiotensin into the lumina of isolated parts of proximal convolutions and measured the flux of sodium and water (a detailed description of this method has been given in the previous chapter, p. 52). By this method the vascular action of angiotensin could be avoided but no tubular effect was observed at such low concentrations, which may be considered physiological. An inhibition of the net sodium flux of about 30 per cent could not be observed until angiotensin had been introduced into the lumen at a concentration of about 10 mg per 100 ml of solution. This negative result, however, did not exclude that angiotensin might have an effect at physiological concentrations when supplied from the peritubular side of the cells. In analogy vasopressin – also an octapeptide – is generally believed to act from the blood side on the water flux and the sodium transport of the distal tubules and collecting ducts by altering the permeability properties of the luminal membrane system. This assumption was adopted after it had been demonstrated by Koefoed Johnsen and Ussing (1953) that the application of vasopressin (antidiuretic hormone or ADH) to frog skin increases the water permeability of the skin if added to the inside solution while no effect was observed when added to the outside bathing solution (net flux from the outside to the inside of the skin). This effect was located to the outward facing membrane (MacRobbie and Ussing 1961) as indicated by measurements of swelling rates of the hormone treated epithelium when swelling was induced by changing the osmolality of the outside solution. Similarly Hays and Leaf (1962) using toad bladder demonstrated that the increased net flux of water from the mucosal to the serosal side induced by adding vasopressin to the serosal medium was accompanied by an increased concentration of tagged water (THO) in the epithelium provided the tagged water was added to the mucosal bathing solution. This result clearly indicates that vasopressin increases the permeability of a barrier located to the mucosal surface of the bladder. Further, the stimulating effect of vasopressin on the sodium transport in the frog skin was shown to be due to a reduction of the partial resistance to the sodium current at some site in the skin whereas the electromotive force of the sodium transporting mechanism remained almost

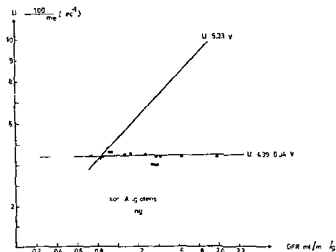


Fig 2 *Reciprocal occlusion times after injections of 25 ng of angiotensin at different clearances of inulin measured immediately before the administration of angiotensin* (Leyssac 1965 b)

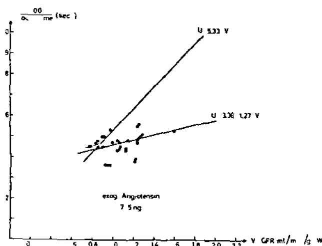


Fig 3 *Reciprocal occlusion times after injections of 7.15 ng of angiotensin at different clearances of inulin measured immediately before the administration of angiotensin* (Leyssac 1965 b)

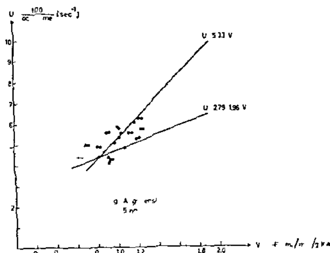


Fig 4 *Reciprocal occlusion times after injections of 5 ng of angiotensin at different clearance of inulin measured immediately before the administration of angiotensin* (Leyssac 1965 b)

The immediate effect of three doses of synthetic angiotension II amide on the reciprocal occlusion time (u) at spontaneously different clearances of inulin (v) is shown in the figures 2–4 An effect is seen to be clearly demonstrated at all three dose levels increasing with the dose administered until a maximum occlusion time (about 23 sec) is approximately reached by the injection of 25 ng of angiotensin This maximum equals the average maximal occlusion time observed spontaneously (Fig 1) which corresponds to an inulin clearance of about 0 85 ml/min/g KW

A quantitative description of the observed tubular effect caused by the injected doses of angiotensin (d_i) at any preexisting rate of reabsorption which must be determined by the equivalent to or a certain but unknown dose of endogenous angiotensin (d_1), is derived from an assumed dose response relationship of the form

$$v(d) = \beta e^{-\gamma d} + \alpha$$

in which α β and γ are positive constants and (d) the total dose of angiotensin (for details see Appendix) By means of linear regression analysis approximate values of α (0 83 ml/min/g KW) and γ (0 2 ng^{-1}) were estimated Using the above dose response relationship the reciprocal of the occlusion times following an injected dose of angiotensin will yield a straight line when plotted as a function of the preexisting clearance of inulin $v(d_1)$

Using the estimated values of (α) and (γ) the observed data are described by the regression lines given in Tabel I and shown in the Figs 2–4 At any pre existing inulin clearance the effect of the injected dose of angiotensin is indicated by the vertical interval between the regression line (u) = 5.33 (v), and the regression line calculated on the basis of the occlusion times observed immediately after the administration of angiotensin

From the above exponential dose response relationship the effect on the occlusion time caused by the three doses of angiotensin as a percentage of the maximally possible effect may be derived (see Appendix) and was calculated to be about 60 per cent, 75 per cent and 99–100 per cent with 5 ng 7.15 ng and 25 ng respectively

v (d_1) \ v ($d_1 = d_2$)	0.85	0.84
1.00	11	14
1.30	16	19
1.60	18	22

Table II *Shows doses of exogenous angiotensin (ng) calculated to reduce Cl_{In} from three spontaneous levels down to 0.85 and 0.84 ml/min/g KW respectively*

In table II is given the calculated amount of exogenous angiotensin (d_2 expressed in units of 10^{-9} g) necessary to reduce the inulin clearance in rat kidney from three different values (v (d_1) ml/min/g KW) to 0.85 or 0.84 ml/min/g KW (v ($d_1 + d_2$)) respectively

Thus the data given in figs 2–4 indicate that under these experimental conditions the maximal effect brought about by exogenous angiotensin is a *primary inhibition of the proximal reabsorption rate to a minimum value* which in rats gives a glomerular filtration rate of about 0.8 ml/min/g KW this is equal to the lowest spontaneous value observed This immediate and direct effect was shown to be rapidly reversible (Leyssac 1964 a) A similar direct inhibitory effect of angiotensin on the proximal reabsorption rate was further demonstrated by *repeated registrations of the proximal intratubular pressures* during the pressure decline induced either by aortic clamping or by intra venous administration of 25 ng of angiotensin (Leyssac 1965 a) The intra

tubular pressure was found to reach zero value within 12–20 sec following complete aortic clamping at which time the lumina were completely emptied of fluid When 25 ng of angiotensin are injected into a rat the vasoconstrictive effect acting also on the afferent vessel (if applied into the systemic circulation) will abolish for a moment more or less completely, the renal circulation (as indicated by the cessation of the red cell circulation in the peritubular capillaries), thus imitating the effect of aortic clamping consequently the glomerular filtration will cease The proximal intraluminal pressure decline following such injection aimed towards zero value in about 20–30 sec as opposed to 12–20 sec seen when no angiotensin was administered the zero value was reached only occasionally because of the very brief vascular effect which generally would allow filtration to be reestablished before the proximal lumina were completely emptied The more slow pressure decline observed in the presence of angiotensin may be taken as a further direct evidence that the rate of proximal fluid reabsorption from the lumen is decreased on account of a primary tubular action

Finally a direct tubular action of angiotensin was also suggested by Langford and Fallis (1963) on the basis of experiments with the Sperber chicken preparation A more detailed report on these experiments was given by Langford (1964) It was shown that infusion of 0 1 γ/kg/min or more of angiotensin via the leg vein to the renal portal circulation of one of the kidneys induced a promptly increased urine flow and sodium excretion rate ipsilaterally Also infusions of noradrenaline (5 γ/kg/min) provoked a prompt diuresis but there was no lateralization and the urinary sodium concentration decreased with increasing urine flow after noradrenaline as opposed to an increased sodium concentration with diuresis following infusions of angiotensin Glomerular filtration rates as estimated by inulin clearance in 5 hens were somewhat instable and no significant effect of angiotensin could be demonstrated These results suggesting a direct tubular inhibition of sodium and water reabsorption in the avian kidney fail to reveal to which tubular section the action might be localized and may be subject to the objection that a vascular action of angiotensin on the afferent and/or efferent arterioles is not excluded by the Sperber technique since these vessels also are surrounded by the peritubular capillaries draining from the renal portal system

A dose of 15–20 ng of angiotensin might at first glance appear to be a rather heavy (unphysiological) dose which could elevate the systemic arterial pressure considerably It should be borne in mind however that the tubular effect is measured only 9 sec after start of the injection at which time only a minor fraction of the injected amount of substance has actually reached the kidney By simultaneous injection of similar doses of pressor substances

with ^{131}J labelled albumin and removal of the kidney 9 sec after start of the injection, it was indicated that only a fraction of 2–4 per cent of the injected amount of albumin had reached the kidney in that time (Leyssac 1965 b). Assuming that also as little as 3 per cent of the injected amount of angiotensin is present in the kidney at the instant of registration of its effect, a maximum effect is obtained when about 0.7 ng of exogenous angiotensin is acting per g of rat kidney, equal to about 1.2 ng (or 1.2×10^{-12} mol or 7×10^{11} molecules) per g of tubular cells (which corresponds to about 60 per cent of the kidney's volume). If the spontaneous variation in the rate of proximal reabsorption of fluid (or salt and water Tm) is further assumed to be elicited by various concentrations of endogenous angiotensin, the data indicate that a difference in the amount of endogenous angiotensin in a rat kidney corresponding to about 7×10^{11} molecules per g of tubular cells may be responsible for the physiological interval of variation in the Tm. The proximal tubules are highly sensitive to a change of this order of magnitude. Therefore the amount of free endogenous angiotensin in the kidney must probably be of the same order of magnitude. With cubic cells of a thickness of 6–7 microns (μ), a change in the order of 200 molecules per cell will be sufficient to elicit a maximum physiological depression of the proximal reabsorption rate, apparently a reasonably low number for a physiologically active hormone. The localization of the renin angiotensin system to the juxtaglomerular apparatus and the demonstrated rapidly reversible and momentary tubular action of angiotensin with its characteristic maximum strongly suggests that angiotensin also exerts its vasoconstrictive action in the same area, which readily might explain the whole physiological range of variation in the renal blood flow as well as in the proximal Tm for salt and water and rate of filtration. *On this basis it may be justified to conclude that the renin angiotensin system is a physiological regulator of these parameters.*

The observation of a characteristic maximum in the tubular inhibitory effect of injected angiotensin represents the greatest challenge to the comprehension also of the integrated function of the kidney. If a similar maximum is also characteristic for endogenously released angiotensin, even an abnormal increase in the release should not be able to depress the rate of filtration below the physiological range of variation (i.e. below a value of about 0.8 ml/min/g KW in rats) by its action on the proximal tubular transfer of fluid. Other – supplementary – factors or mechanisms has to be postulated to explain such further reductions in the inulin clearance observed under pathological as well as under various experimental conditions. By application of rather tight Goldblatt silver clamps to renal arteries of rats in acute experiments by which the arteries were partially occluded to different degrees depending on the

size of the rat examined, it was demonstrated as mentioned in the preceding chapter (p 66–67) that the reciprocal of the occlusion time had reached a fixed minimum level almost identical to that obtained by injections of 25 ng of synthetic angiotensin and equal to the lowest value seen spontaneously (Leyssac 1964 a Fig 2) The data obtained under this condition of partial arterial clamping indicate directly that the depression of the proximal reabsorptive capacity only can account for a depression of the turnover rate to a value of about 0 8 ml/min/g KW The measured clearances of inulin were reduced far below this value (occasionally down to below 0 1 ml/min/g KW) Other mechanisms therefore had to be looked for It was observed in the same study that the urine flow decreased in direct proportion to the abnormal reduction in inulin clearances below 0 8 ml/min/g KW in contrast to the fact that such correlation was non existing within the physiological interval of variation in accordance with the general experience Moreover, since urine osmolality decreased – approaching isosmolality – pari passu with decreasing urine flow in this low and abnormal interval the observation suggested that the further reduction in clearances of inulin under these conditions was caused by proximal luminal occlusion of more and more nephrons Such luminal occlusion of a varying number of nephrons was directly demonstrated in kidneys frozen at states of function characterized by clearance values below 0 8 ml/min/g KW in contrast to wide open lumina of all nephrons in kidneys frozen at a physiological state of function ($Cl_{in} \geq 0$ 8 ml/min/g KW) These results might suggest indirectly that endogenous angiotensin also exhibits a characteristic maximum in its tubular action and that a depression of the proximal reabsorptive capacity due to angiotensin cannot exceed that observed in the clipped kidneys even if the release of angiotensin is abnormally increased in such kidneys as may be assumed although it has not yet been conclusively proven

A final conclusive demonstration that the release of angiotensin actually varies in inverse proportion to the proximal Tm for salt and water and renal blood circulation within the physiological interval of variation and that the release is abnormally increased in partially stenosed kidneys would definitely confirm the above conclusions It therefore should be considered an important object for future studies to elaborate a sufficiently sensitive analysis for angiotensin permitting a quantitative determination of such minute variations as are prone to occur physiologically in the renal capillary or venous blood Until this has been achieved the most reasonable concept which satisfactorily may explain the available observations, seems to be that physiological variations in the renal blood flow and the proximal reabsorption rate (and thus in the glomerular filtration rate) are mediated by changes in the release of angio

tensin Further (abnormal) reductions in the renal blood flow and filtration rate are due to lowered arterial and glomerular propulsive pressures whereby the number of nephrons participating in urine formation gradually is reduced In view of this concept it may be predicted that the glucose Tm (Tm_G), as inferred from clearance studies apparently would be decreased at such low pressures In accordance herewith this was also observed by Kruhoffer (1950) using dehydrated rabbits, in which Tm_G changed in parallel with clearances of inulin below values in the latter of about 10 ml/min whereas no change in Tm_G was observed above this level The term apparent decrease in Tm_G has been used here because the calculated reduction in the amount of glucose reabsorbed per unit of time in accordance with Kruhoffer s own interpretation is taken as an indication that only the number of active nephrons was reduced by occlusion without necessarily implying that the cellular capacity of the transporting mechanism had changed

The disclosure of the two primary renal effects of angiotensin, viz the vasoconstrictive and the tubular effects satisfactorily explain the established interrelationship of renal blood flow, proximal reabsorption rate, and glomerular filtration rate under physiological variations as due to the regulation by one common factor, and opens the interesting question with which parameter the regulation is primarily concerned

CHAPTER V

A A REGULATED PARAMETER

On the basis of the foregoing discussion it may be accepted that physio logical changes in the renal blood circulation and in the functional states of the proximal segment of mammalian nephrons, estimated by the rates of proximal fluid reabsorption (or turnover rates) of the integrated nephron population are mainly at least reflections of a regulated release of angio tensin from the juxtaglomerular apparatus of the individual nephrons However these investigations have failed to provide an answer to the question whether and how, the juxtaglomerular apparatus may serve as a link by which the functional states of the proximal and distal segments become mutually interrelated

The absolute necessecity of a functionally adjusting link is obvious from the Tm limited transfer rate also of the fraction (80 %) of the filtered volume which is reabsorbed proximally The problem to be faced appears from the general biological requirement of salt balance which in mammals is maintain ed exclusively by a regulated renal salt excretion The renal salt excretion rate must therefore be quite *uninfluenced* by factors unrelated to the salt balance factors which primarily may change either the tubular reabsorption rate (metabolic changes) or the rate of filtration of salt (changes in the glomerular propulsive pressure brought about mainly by changes in pre glomerular vascular resistance)

How this delicately adjusted salt excretion rate may be brought about in the mammalian kidney in spite of these disturbing influences can hardly be understood on the basis of discussions of conditions for a regulation of salt excretion by the integrated kidney since the integrated renal function involves too many unsolved and highly complex problems because of the anatomical and probably also functional inequality of the nephron population in kidneys of various species especially regarding the cortical and juxtamedullary nephrons and their unanalysed relative participation in the excretion rate of salt

Consequently the discussion should be restricted to include considerations on the conditions necessary for a regulated salt excretion rate by the single nephron In this context the suggestion of a small and limited distal reabsorp-

tive capacity of sodium, as inferred from the study by Wesson, Anslow and Smith (1948) on osmotic diuresis (cf Ch I, p 20) seems to be of relevance, and has been confirmed also in recent micropuncture studies by Giebisch, Klose and Windhager (1964) These authors showed that the fraction of filtered load of sodium reabsorbed along the distal tubule, as indicated by the F/P sodium divided by the F/P inulin plotted against the sites of collection in this segment was greatly reduced in hypertonic saline loaded rats as compared with conditions in normal control animals, calculations on the basis of the simultaneous percentile increments in plasma sodium concentration and filtration rates showed that the absolute rates of reabsorption in the distal tubules either had increased to a minor degree remained unchanged, or frequently had slightly reduced under these loading conditions in which the sodium and water excretion rates were markedly elevated Furthermore it is generally agreed now that mineralocorticoid hormones play a dominant role in the physiological regulation of salt excretion by enhancing the distal transfer processes particularly those responsible for an exchange of sodium with other cations This assumption of a distally located site of action is generally substantiated by stop flow experiments by Vander et al (1958) and Vander Wilde and Malvin (1960), who demonstrated that the level of the sodium concentration in the early samples representing fluid from the distal segments is much higher in normal control dogs than in adrenalectomized animals on administration of aldosterone this distal tubular defect may become normalized But as this effect of mineralocorticoid hormones on the sodium excretion rate is considerably delayed (45–60 min), as repeatedly demonstrated, first on extracts of whole adrenal cortex (e g Roemmelt, Sartorius and Pitts 1949), and as the distal transfer capacity for sodium ($Tm^d{}_N$) probably is relatively small and limited it follows that the significance of aldosterone as a regulator of salt excretion must be conditioned by a fairly constant load of sodium to the distal part of the nephrons Finally as the volume of fluid supplied to the distal tubules is determined by the difference of the hydrostatic pressure at the end of the proximal segment and the beginning of the distal tubule and also by the resistance to flow across the loop of Henle which resistance can hardly be actively changed (abscense of muscular cells) it must be presumed that the proximal intratubular pressure has to be kept fairly stable by some intrarenal regulating mechanism In accordance with this argumentation it should be mentioned that Gottschalk and Mylle (1956) showed that the mean proximal intratubular pressure of 13 5 mm Hg (119 recordings in 56 rat kidneys) varied within a range of 7 to 21 mm Hg with a standard deviation of only 2 4 mm Hg and that the average pressure in the proximal lumina in each rat was the same (13 3±3 mm Hg) Similar values were

obtained in control kidneys under similar experimental conditions (Leyssac 1964 b) besides pressures in single proximal convolutions of one kidney were seen to vary within a similar range of about 9 to 20 mm Hg from one area of the surface to the other at any particular time Such fairly relative con stancy of the mean proximal intratubular pressure from one kidney to the other and in the individual kidneys at different times within a pressure range, in which the tubules have more or less the character of rigid tubes (cf preceding chapter p 73–75), might suggest that the intratubular pressure in the single nephron actually is controlled or stabilized by a regulating mechanism Experimental evidence supporting this suggestion was presented also by Leyssac (1964 b), who showed in acute experiments that the application of a rather tight partial constriction (Goldblatt clamp) of the renal artery which reduced the inulin clearance to values far below the normal range (<0 8 ml/min/g KW) did not influence to measurable degrees the proximal intratubular or peritubular capillary pressures whereas the distal intratubular pressure was seen to be reduced significantly Direct evidence had been presented (cf p 85) that the abnormally reduced inulin clearance in such kidneys is due to a cessation of the luminal fluid flow and subsequent luminal occlusion of the proximal convolutions attributable to a more or less complete proximal reabsorption of the filtered fluid provoked by the reduction in the glomerular propulsive pressure following partial arterial clamping in a state of fixed proximal Tm for salt and water Thus the glomerular capillary hydrostatic pressure in these kidneys must have been significantly lower than normal The proximal luminal pressure of still functioning nephrons remained unchanged It therefore seems safe to conclude *that the proximal intratubular pressure is subject to a very efficient regulating mechanism* and that the fair constancy of the mean proximal intratubular pressure from time to time and from one normal rat to the other is a reflection of this regulation Since further it may be assumed that these normal (control) rat kidneys have represented the greater part of the entire range of spontaneous variations in the salt turnover rate and blood flow it might be tempting to relate also the relative constancy of pressure to these variations in other words tentatively to suggest (or infer) that the physiological changes in the release of angiotensin by its vascular and tubular effects were responsible for the regulation of the proximal intratubular pressure

It may be questioned whether it is essential to know exactly the magnitude of the pressure decrement across the glomerular membrane in order to accept that the tubular action of angiotensin plays a significant part in the regulation of the proximal intratubular pressure But such knowledge is hardly of any importance as may emerge from the following argumentation Thus 1) if there

is only a moderate resistance to flow across the membrane a greater rate of filtration must be conditioned by greater pressure decline (i e greater effective filtration pressure) If this resistance is inferior to the resistance offered by the distal segments of the nephron (viz from the beginning of the loop of Henle to the renal pelvis), in conformity with previous conclusions (p 70–71), any change in filtration rate i e in effective filtration pressure must be determined by changes in the rate of proximal reabsorption (Tm^{P}_{N}) which primarily changes the intratubular pressure Conversely changes in the glomerular propulsive pressure will involve primarily an almost parallel change in the proximal, intratubular pressure but no perceptible change in the filtration rate (cf p 71) Realizing quite well that it has never been verified in experiments it might serve as a hypothetical example that the extremes of the normally occurring variations in the proximal intratubular pressure (7–19 mm Hg) might represent extremes of an 'incompensated state, viz variations of about 6 mm Hg to each side of the compensated mean pressure of 13 mm Hg This variation would also represent the *maximum* change in the effective filtration pressure, since regulated changes in the reabsorption rate induced by endogenous angiotensin normally will be accompanied in all probability, by changes in the tone of the efferent arteriole (cf discussion below, p 104–105) and thereby in the glomerular capillary hydrostatic pressure which will change in the same direction as the intratubular pressure change elicited by the changed reabsorption rate Consequently in rats a change in the filtration rate from 0 8 to 1 6 ml/min/g KW (normal range of variation) should correspond to a change in the effective filtration pressure not exceeding 6 mm Hg On the assumption that the rate of filtration is linearly dependent on the effective filtration pressure 0 8 ml/min/g KW becomes filtered at an effective filtration pressure of 6 mm Hg 1 6 ml/min/g KW being filtered at a pressure of 12 mm Hg i e declines in the pressure across the glomerular membrane ranging at levels not exceeding 6 to 12 mm Hg determined by the rates of proximal reab orption

2) If on the other hand the pressure drop across the glomerular membrane is considered to be small as compared with the oncotic pressure (but still with a proximal transfer of salt of Tm-character), an increase in the glomerular propulsive pressure would still be accompanied by an almost parallel increase in the proximal luminal pressure and a proportional increase in the rate of excretion of salt and water A pressure regulation by which the proximal reabsorption rate *is not* simultaneously changed i e mediated solely by an efferent arteriolar relaxation would then involve an increased renal blood flow and since the filtration rate had not changed significantly, a reduction in the filtration fraction (GFR/RPF = FF) Consequently the final

glomerular oncotic pressure will be below the normal level and the glomerular propulsive pressure and proximal intratubular pressure will remain elevated in spite of a normalized capillary hydrostatic pressure These two effects of an efferent arteriolar relaxation apparently will counteract each other and changes in the oncotic pressure become highly significant to the intratubular pressure Thus, in order to maintain the proximal luminal pressure unchanged the glomerular hydrostatic pressure should overcompensate exactly to a degree at which it counteracts the change in oncotic pressure This will be found to be a highly vulnerable regulating mechanism, since it has to rely solely on the control of the resistance offered by the one efferent arteriolar vessel In addition the vascular resistance to flow being determined by the internal radius in the fourth power such intratubular pressure regulation would require an extremely delicate and exact adjustment of the arteriolar tone this regulation would be far less critical if the tubular reabsorption also were to play a significant part in the pressure regulation However angiotensin is found by now to have actually a tubular action, by which the filtration rate does change in such a way that changes in the filtration fraction are avoided The inference hereof is that the tubular action also in this case has a stabilizing (or regulating) effect on the proximal intratubular pressure

B CONDITIONS OF CHANGED RELEASE OF ANGIOTENSIN OR RENIN

The question to emerge next is whether or not the above hypothetical concept of changes in the release of angiotensin being brought about in order to regulate the proximal intratubular pressure is compatible at all with the results obtained from investigations on the adequate stimuli(us) causing a release of angiotensin or renin It has been shown unequivocally in several laboratories that angiotensin (in the sense of the octapeptide angiotensin II) is the active substance but the analytical methods remain to be perfected to a degree of sensitivity at which it might be established whether the juxta glomerular apparatus under psysiological conditions releases angiotensin and/or renin directly into the blood circulation in which angiotensin may be formed instantaneously by renin reaction with the plasma substrate (angiotensinogen) The presence of renin also in normal human and rabbit plasma as indicated by recent investigations reported by Peart s group in London (Brown et al 1963 b, Lever and Tree 1963) who used the best and for the time being most sensitive renin assay strongly suggests a direct release of

renin into the blood Whether this is true also for angiotensin remains to be established But the degree of correlation is fair, apparently, of changes in plasma concentrations of renin and angiotensin under a variety of abnormal conditions and of such changes in the plasma concentration and in the renal renin content, hence it seems to be justified to discuss collectively these studies

a) *Renal renin content in kidneys in which the release of renin and/or angiotensin assumedly is increased*

Numerous studies of the renin content in kidneys and plasma have been published since the time when Goldblatt and collaborators (1934) demonstrated in dogs that arterial hypertension might be provoked by partial clamping of the renal arteries In brief the renin content has been estimated by several types of bio assay either from crude or from more or less purified saline extracts of kidneys either directly, as renin, or by the indirect test, by which renin is incubated with substrate under standard conditions and the yield of formed angiotensin is estimated It is outside the scope of this review to enter into a detailed discussion about the many objections raised to the different types of assay preparations, only it shall be mentioned that by now it seems generally accepted that intravenous injection of the test solution into a normal or nephrectomized rat or other mammal and subsequent registration of the increase in the blood pressure (dog test or rat test) is the bioassay which is most reliable and hence the one of choice It should be briefly mentioned also that the only reliable results to be obtained by the indirect renin test (estimating the formed amount of angiotensin) originate in studies in which the inactivation of angiotensinase prior to incubation may be regarded as complete as emphasized by several authors (e g Kemp 1962) Results obtained by the direct test in which crude extracts are injected should be taken also with a certain reservation because the presence of depressor material may represent a severe source of error An additional although less likely source of error involved in this method might be a difference in the renal content of angiotensinase indications of which were seen by Bing (1962 a) in clamped kidneys versus control kidneys The formation and pressor action of angiotensin being extremely rapid however this error will probably be insignificant In view of these many sources of error it can hardly surprise that a rather complex and often contradictory picture is obtained from the earlier literature and consequently a few only of the most thourough investigations using the most reliable methods for the identification of the pressor substance either as renin or

angiotensin shall be included here any further comments on the particular procedures of identification are not given

Pickering Prinzmetal and Kelsall (1942) used the direct renin test of an extract of rabbit kidneys and found that the renin content had increased as compared with normal in kidneys during the first week after the application of a partial clamp to the renal artery In rabbits in which chronic hypertension (Goldblatt hypertension) persisted for 2–6 months the renal renin content was normal Similarly Haas and Goldblatt (1959) showed in dogs that the renin content was about 2–8 times greater in the clipped kidney than in normal kidneys 13–30 days after application of the clamp and further, that the renin content was reduced in the untouched, contralateral kidney Also in hypertensive patients they found an increased renal renin content as compared with normal human kidneys Unfortunately it is not reported whether or not these patients were renal hypertensives nor whether the hypertension was of a benign or malignant nature The conclusion drawn from these results has been further established by Bing (1962 b) in experiments on rats in which one renal artery was partially clamped and is in general accordance with results obtained in several other investigations Further it has been substantiated by observations of parallel changes in the number and intensity of staining of the granules of the juxtaglomerular cells (the juxtaglomerular index or JGI) since it has been reported by Tobian Janecek and Tomboulain (1959) that the JGI as well as the pressor substance activity was increased in the partially clamped kidney but decreased in the contralateral untouched kidney An increased pressor activity most likely being due to renin was observed also in adrenalectomized rats by Gross and Sulser (1957) whereas pressor activity and JGI were reduced in kidneys of rats treated with desoxy corticosterone acetate (DOCA) and salt (Tobian Janecek and Tomboulain 1959) or according to Tobian (1960) by heavy peroral salt loading alone It should be emphasized however that although later investigations to be referred below strongly have suggested that an increased release of renin and/or angiotensin is not infrequent in such clamped kidneys in which the renin content and/or concentration have increased it has not been established whether this is *always* the case and the mere finding of a change in the renal renin content makes it hardly justified to infer that a parallel change in the release has also occurred This fact was further emphasized in the discussions in Quebec where Hartroft (Hartroft Sutherland and Hartroft 1964) declared that degranulation of the juxtaglomerular cells may occur as an acute phenomenon following immediately upon sodium depletion the latter always being followed by increased granularity provided the sodium depletion is maintained Consequently a greater significance should be attached to findings

recently obtained in studies on the renin and angiotensin concentrations in lymph and plasma the experiments were carried out under varying conditions and, the methods by which the findings were obtained may be characterized as reasonably safe and reliable These results have directly confirmed the assumption of an increased renin release under conditions of bleeding shock and within the first weeks after partial constriction of the renal artery, and have gained a further indirect support by the demonstration that other enzymes also may be released from the kidney under such conditions Kemp (1960) has presented evidence that lactic acid dehydrogenase is liberated to the renal venous blood of the clamped kidney in the early phase of Goldblatt hypertension in rabbits and Kemp and Laursen (1962) demonstrated in cats an increase in serum lactic acid dehydrogenase (LD) – and glutamic oxalacetic acid transaminase (GOT) activity during severe bleeding hypotension In a few cases they showed by electrophoresis that some of the LD activity could be ascribed to renal isoenzymes of LD

b) *Changes in lymph and plasma concentrations of renin and angiotensin*

Even before the renin assay introduced by Peart s group had been perfected to a sensitivity at which renin could be demonstrated in the renal venous blood Lever and Peart (1961) had been able to demonstrate an increase in pressor material in lymph collected from the thoracic duct of dogs in which the renal artery was partially constricted The pressor substance was identified as renin

A similar increase in renin concentration in thoracic duct lymph has recently been reported by Higgins Davis and Urquhart (1964) using dogs with experimental, secondary aldosteronism and ascites provoked by thoracic cava inferior constriction After bilateral nephrectomy the pressor activity was reduced in such dogs

Similarly Davis and his group (Higgins Davis Urquhart and Olichney 1964) reported an increase in the renin concentration in plasma of anaesthetized as well as conscious dogs with thoracic cava inferior constriction Concentrations of renin were considerably higher in the renal venous plasma than in the peripheral plasma and about 2 5 times higher than in blood drawn from normal drogs Further following nephrectomy the activity in the peripheral plasma was seen to decline in all cases finally they presented evidence that an excess of plasma angiotensinogen was present in equal amounts both in normal control and experimental animals and consequently it could not represent the limiting factor to the formation of angiotensin in their indirect assay

A series of investigations carried out under several conditions and concerned with the concentrations of renin and angiotensin in plasma were reported at the international symposium on angiotensin sodium and hypertension held in Quebec October 1963, a few of these investigations shall be referred Scornic and Paladini (1964) assayed preformed angiotensin in arterial plasma of dogs and found significantly increased plasma concentrations after severe bleeding (arterial pressures of 80 mm Hg and below), in nephrectomized dogs bleeding provoked no such effect by which the early observation by Huidobro and Braun Menendez (1942) is confirmed These authors found also an increase in the plasma concentration of angiotensin in acute Goldblatt hypertension after tight partial clamping (in chronically hypertensive dogs such increase was not seen) in salt depleted animals and after infusion of 2–4 μg of noradrenaline per kg per min, by which the systemic arterial pressure was elevated by 20–40 mm Hg However, such increase could not be demonstrated after aortic clamping above the renal arteries by which the femoral arterial pressure was reduced to between 60 and 35 mm Hg whereas a combination of the aortic constriction and noradrenaline infusion (2–4 μg/kg/min) was seen to elevate the peripheral angiotensin level This apparent failure to increase the plasma angiotensin concentration after aortic constriction alone (indicated by their method) as opposed to conditions of bleeding and noradrenaline infusions might be due in part to the fact that these kidneys were not denervated A compensatory, arteriolar vasodilation in response to aortic constriction was not excluded the consequence of which might have been that the pressure at the end of the afferent arteriole or the glomerular propulsive pressure had not reduced to nearly the same extent as during bleeding shock and noradrenaline administration Unfortunately renal blood flow inulin clearance and sodium excretion rate were not estimated in these experiments Their negative finding under conditions of aortic constriction is in contrast to findings by other investigators (e g Skinner McCubbin and Page 1963 cf p 97) who found levels of angiotensin like pressor material to be increased in the renal venous plasma only few minutes after reduction of the mean perfusion pressure to the kidney In analogy Vander and Miller (1964) observed increased levels of renin in the renal venous plasma after similar constrictions of the aorta above the renal arteries Their study shall be discussed later in greater detail The reason for the discrepancy between these results seems uncertain The kidneys were not denervated either in any of these latter investigations but it should be mentioned that Scornic and Paladini only tested pressor substance in the peripheral plasma not in renal venous plasma as was the case in the other studies

recently obtained in studies on the renin and angiotensin concentrations in lymph and plasma, the experiments were carried out under varying conditions and the methods by which the findings were obtained may be characterized as reasonably safe and reliable These results have directly confirmed the assumption of an increased renin release under conditions of bleeding shock and within the first weeks after partial constriction of the renal artery and have gained a further indirect support by the demonstration that other enzymes also may be released from the kidney under such conditions Kemp (1960) has presented evidence that lactic acid dehydrogenase is liberated to the renal venous blood of the clamped kidney in the early phase of Goldblatt hypertension in rabbits and Kemp and Laursen (1962) demonstrated in cats an increase in serum lactic acid dehydrogenase (LD) – and glutamic oxalacetic acid transaminase (GOT) activity during severe bleeding hypotension In a few cases they showed by electrophoresis that some of the LD activity could be ascribed to renal isoenzymes of LD

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centrations in plasma of these two substances under a variety of abnormal conditions the oversimplification may be accepted at present for the sake of conceptual approach that the release of renin and/or angiotensin under these experimental and pathological states may be considered as caricatured illustrations of a physiological regulating mechanism in which angiotensin is the active factor In order to simplify the following discussion an increased release of renin and/or angiotensin will be considered equal to an increased release of angiotensin

It is apparent from the literature *that the release of angiotensin is stimulated* under conditions of salt depletion Addison s disease cardiac in suffiency cirrhosis and other states involving oedema in secondary experi mental aldosteronism due to thoracic cava inferior constriction bleeding shock administration of pressure substances (nor adrenaline), and not in frequently after partial constriction of the renal artery or the aorta above the renal arteries in the latter cases probably depending on the degree of arterial obstruction *alltogether conditions which have in common a tendency to or a reduction in the glomerular propulsive pressure* This has been further established by Skinner, McCubbin and Page (1963) by partial obstruction of the renal circulation They observed an increase in the renal venous plasma of a pressor substance, most likely being angiotensin only 5 min after minor reductions in the mean arterial pressure Obstructions giving identical reduc tions in pulse pressure and renal plasma flow but slighter changes in the mean arterial pressure failed to cause any detectable increase in the release of the pressor substance

Before entering into the discussion whether and how these observations fit with the present concept of angiotensin as a regulator of the proximal intra tubular pressure a brief comment shall be made concerning some of the previous hypotheses on the adequate stimulus to the juxtaglomerular apparatus and its possible physiological significance to the renal function

C HYPOTHESES ON THE STIMULUS TO ANGIOTENSIN RELEASE AND/OR THE FUNCTION OF THE JUXTAGLOMERULAR APPARATUS

Only a few of the previously proposed hypotheses shall be discussed here the intention being to present only representatives of current ideas on the function of the juxtaglomerular apparatus and the adequate stimulus(1) to release of angiotensin This topic should not be discussed however, without

mentioning that several anatomists and physiologists have considered and still believe that any physiological function cannot be attributed to this structure which is believed to be a degenerated or passively (mechanically) modified, group of cells

Since Goormaghtigh (1940) suggested that renin might be secreted from these cells several investigators have contributed to our present knowledge of the structural and ultrastructural characteristics of the different types of cells of the juxtaglomerular apparatus and on purely morphological evidence have proposed theories as regards the possible stimulus to the renin angiotensin system Thus McManus (1947) emphasized that while the Golgi apparatus of all other cells of the nephron is localized to the luminal side of the nucleus it is placed in the basal side of the macula densa cells and, thus close to the other constituents of the juxtaglomerular apparatus This localization of the Golgi apparatus was supposed to be indicative of a secretory activity towards the other cells of the structure (cf Bing s concept mentioned p 77) Furthermore McManus pointed out that the basement membrane of the macula densa cells is extremely thin or even interrupted towards the network of Goormaghtigh cells suggesting that fluid absorbed by the macula cells of the distal tubule easily and without restriction might diffuse between the different parts of the juxtaglomerular apparatus In accordance with this Oberling and Hatt (1960) confirmed by electronmicrography the discontinuity of the basement membrane of the macula densa cells through which membrane these cells are in intimate contact with the lacis or cytoplasmatic network of the adjacent cells suggesting that the composition of the distal tubular fluid in some way might influence the release or production of renin The same suggestion viz that distal fluid osmolar or sodium concentration via the macula densa cells is an important signal to a regulation in some way of urine formation was also advocated by Latta and Maunsbach (1962) who in a study by electron microscopy pointed out the existence of extracellular compartments (vacuoles) at the base of these tubular cells as morphological evidence suggesting fluid transport from the tubular lumen to the other cells of the juxtaglomerular apparatus Also the occasional observations of an apical swelling of the macular cells were taken as evidence of their osmotic sensitivity Obviously other interpretations are possible Although these structural observations are insufficient and at least fail to suggest how a possible change in the distal fluid composition might influence the angiotensin release or renin production and how this again might influence renal function and sodium balance they do stress the obvious relevance of the anatomical interrelation of the distal tubule the arterioles and the Goormaghtigh cells

In the preceding section (B) a report is given of the evidence provided

that the release of angiotensin is stimulated under conditions of negative sodium balance and other conditions which in this respect imitate a decreased extracellular fluid volume or distension of the vascular system on the basis of the ample evidence collected in support hereof Tobian (1960) suggested that a decreased stretch of the juxtaglomerular cells of the afferent arteriole is actually the stimulus to the renin angiotensin system But whether or not such release were of significance to the renal functional parameters was not explained except that it might exert some influence on the adrenal mineralocorticoid secretion thus causing sodium retention by which the extracellular volume would be restored Tobian s hypothesis is said to be supported by the finding in normal rabbits viz that the juxtaglomerular zone of glomeruli situated near the origin of an interlobular artery contains little or no renin while that of glomeruli closer to the capsule of the kidneys were rich in renin (Brown et al 1963 a) suggesting that this distribution of renin content might be related to the arteriolar pressure, if variations in the renin content indicate parallel variations in renin secretion (Brown et al 1964 b) However their results indicate merely that the renin release may be stimulated under conditions of low arteriolar pressure, but fail to present evidence of whether the stimulus actually is an arteriolar stretch or some other parameter such as the glomerular propulsive pressure or some function thereof which under these conditions will be similarly changed Hence the inference that such data are in support of Tobian s theory as such is rather unjustified Schmid (1962) extended the main idea that arteriolar stretch or pressure might be a stimulus to the release of angiotensin He advanced the theory of a balance mechanism operating across the glomerular tufts between changes in the afferent arteriolar pressure at the site of the juxtaglomerular cells and changes in the post glomerular resistance elicited by angiotensin The juxtaglomerular cells were assumed to be sensitive to pressure and to increase their release of angiotensin at reduced pressure at this site An increased efferent resistance as a consequence of the release of angiotensin would thereby increase the intraglomerular pressure Further the glomerular capillaries were presumed to constitute the return loop of a feed back mechanism to the pressure sensitive juxtaglomerular cells In effect this mechanism would serve to maintain a constant filtration pressure according to Schmid (cf discussion p 89–91) These ideas of a decreased stretch or pressure and modifications thereof as an adequate stimulus (e g suggestions of expansile pulsation or dynamic compliance as proposed most recently by Lowe (1964)) disregards the highly suggestive importance of the junction between the distal tubule and the other constituents of the juxtaglomerular apparatus and seem to disagree with observations by other authors

In this context attention should be directed to the interesting study reported by Sellwood and Verney (1954–55) In very careful clearance studies on the effect of oral water and saline administration in dogs they demonstrated that increases in the renal plasma flow and to a minor degree, in the glomerular filtration rate following water loading (2 5–3 0 per cent of body weight) exceeded the increases observed after loadings with 0 875 % of sodium chloride (2 4–3 0 per cent of body weight) These changes were independent of changes in the arterial pressure, actually this pressure was not seen to change during experiments in which the animals had been subjected to complete, or abdominal sympathectomy Quite logically the authors argued that, if the increased plasma flow were the result of a reduced viscosity of the blood or an expansion of the glomerular capillaries (or as they might have included of the efferent vessels) accompanying the increase in blood volume it might have been expected – in contrast to their observations – that these parameters were changed more efficiently by saline loading than by water loadings Based on this evidence they inferred that changes in the renal plasma flow and filtration rate are of intrinsic renal origin Besides these increases preceded the increase in urine flow, occasionally decreasing again before the rate of urine flow had reached its peak suggesting that the increases are associated with the induced changes in the water load On the assumption that the increase in water content of the blood is not the primary (direct) cause of a fall in resistance of the arteriolar vessels we are driven to conclude that the slight increase in filtration pressure and therewith in filtration flow is the factor which is primarily responsible for the change in vascular resistance expressed in the observed increase in plasma flow We infer therefore, that an initial increase in pressure within the tubule – acting in some unknown way and at an undetermined site – causes relaxation of the afferent arteriole and therewith a rise in glomerular capillary pressure The initially effective increase in intratubular pressure must on this hypothesis be extremely small The authors actually surmised that the macula densa region hypothetically might be the site in the tubule which through changes in the luminal pressure is controlling the afferent vascular tone this teory was abandoned again because antidiuretic hormone which assumedly reduces the distal intratubular pressure if administered to animals in water diuresis has no appreciable effect on the renal plasma flow or filtration rate It seems rather impressive how close to the present problem these authors arrived basing their deductions merely on data obtained in their clearance study The suggestion that a vasodilator substance might be acting on the pre glomerular vessel is evidently based upon the (falsified) concept that the filtration rate is determited exclusively by the glomerular propulsive pressure According to the present

concept the increase in the filtration rate and plasma flow observed as an event secondary to the increased intratubular pressure or some function thereof might equally well be caused by a decreased release of angiotensin As observed, renal blood flow the proximal Tm for salt and water and consequently the filtration rate will then increase

Further evidence of the stimulus to the renin angiotensin system has recently been reported by Vander and Miller (1964), whose results seem to be an important contribution to our present knowledge on this topic, their indirect renin test may be subject to some objections though In this respect the greatest importance should be attached to the fact that the incubated partly purified plasma samples and substrates used here apparently were not completely free from angiotensinase since a loss of about 25 per cent of added synthetic angiotensin was observed during incubation Further although probably of minor significance the amounts of angiotensinogen in the incubation media were not chequed However each one of these errors if any are prone to reduce the differences observed in plasma renin concentration of the renal venous blood in control and experimental periods In the interpretation of their data it is of great importance that all results were obtained in acute experiments thus minimizing the influence of late secondary adaptive mechanisms

The renal venous plasma pressor activity detectable in 85 per cent of normal dogs was correlated to renal plasma flow(determined by the Fick principle using para amino hippurate (PAH) creatine clearance urine flow sodium plasma concentration – and excretion – and systematic arterial pressure In control animals the renin concentration and arterial pressure renal plasma flow plasma sodium concentration and filtration fraction were not seen to be correlated Some inverse relationship was suggested however between the renin level and creatinine clearance although this correlation was statistically insignificant (corr coeff – 0 24 P 0 2–0 1), but a significant inverse relationship was found between the renin activity and the sodium excretion rate (corr coeff – 0 38 P $\leqq$ 0 04) Taking into account the rather narrow range of variation in the creatinine clearance (1 67±0 43 ml/min/kg BW) relative to that of sodium excretion rate (1 5±1 6 μM/min/kg BW) the relatively great variability involved in the clearance determination as opposed to the exactness and reproducibility of determinations of sodium excretion rate as well as the rather insensitive bio assay method used it can hardly surprise that correlation was not found significant between the renin plasma concentration and turnover rate of salt and water

Renal venous plasma pressor activity increased significantly in 10 min

after partial constriction of the aorta above the renal arteries by which the mean femoral arterial pressure was reduced to 90 mm Hg The activity was maximal 20–30 min after clamping and decreased again 10 min after re opening of the arterial clamp If however, diuresis was induced during aortic clamping, either as an osmotic diuresis (mannitol, urea or sodium sulphate) or by administration of chlorothiazide or azetazolamide, the increased pressor activity decreased again to pre clamping values but neither plasma flow nor the creatinine clearance did return (increase) to control levels If on the other hand the aorta was constricted during diuresis the increase in the pressor activity in the renal venous plasma always observed after clamping in non diuretic animals, was eliminated The diuretics however did not pre vent the reductions in plasma flow or turnover rate of salt Finally significant increases in plasma renin levels were found to follow high ureteral pressure (at least 60 mm Hg), induced by injection of urine into the renal pelvis via an ureteral catheter

These results strongly support the view that a decreased pre glomerular arteriolar pressure or distension per se is not the major stimulus to the renin angiotensin system The possibility that the pre glomerular resistance in clamp ed animals might have been reduced by the diuretics, the effect being a compensatory increase of the distension (causing a reduced renin secretion) seems less likely since the renal plasma flow in these animals did not increase during diuresis Vander's conclusion that the renin secretion is stimulated by a pressure reduction affecting some other functional parameter therefore seems justified He further argues that the intratubular pressure or distension is not likely either to be the stimulus since renin output had been decreased by osmotic and other diuretics known to elevate this pressure but was in creased after application of high ureteral pressure which would have a similar effect on tubular pressures Thus according to Vander the most probable although hypothetical conclusions to be drawn from his results seems to be that the renin angiotensin system is controlled by the flow or the composition of the distal intratubular fluid probably at the site of the macula densa Although his results admittedly are suggestive of this conclusion it should be emphasized that the applied intratubular pressure during ureteral occlusion (from the study by Gottschalk and Mylle (1956) known to be parallel with renal pelvic pressures at levels above the normal intraluminal pressure of the distal tubules) was extremely unphysiological the intrarenal vascular resi stances and distribution of pressure declines may also have been significantly disturbed under this condition whereby one experimental condition may become related to another but possibly uncomparable situation Hence it

cannot be excluded completely that a normal response of the juxtaglomerular apparatus may have become reversed under this extreme condition

To summarize the many direct and indirect observations here presented concerning the conditions of a changed renin and/or angiotensin release it may be stated in conclusion that we remain ignorant of the adequate stimulus to the juxtaglomerular apparatus, but apparently changes in the afferent arteriolar pressure or distension can on no account be considered the major stimulus which rather may be some function of a change (reduction) in the glomerular propulsive pressure, whether this be the (reduced) flow the composition of the tubular fluid or – not very likely – the intratubular pressure or distension at the site of the macula densa

Vander and Miller (1964) did not suggest whether or how the resulting intrarenal actions of the liberated angiotensin might have a regulating feedback effect on the releasing stimulus In this respect an additional comment should be made to their observations It might be adequate to ask whether the present concept viz that normal variations in filtration rate are determined by the tubular inhibitory action of angiotensin (increased release causing reduction in the filtration rate) has been invalidated by their observation that the glomerular filtration rate did not increase after the fall in plasma renin concentration after diuresis induced in partially clamped kidneys This objection does not seem reasonable however since it is most likely that the proximal tubular reabsorption rate at the same time may have been inhibited by the osmotic action (creating a rate limiting concentration gradient) in case of osmotic diuresis and by a possible direct or indirect inhibitory tubular action of chlorothiazide and azetazolamide when these drugs were given

A few of the more recent hypotheses on the function of the juxtaglomerular apparatus deserve a critical discussion Thus Swann (1961) advanced the theory that the kidney balloon is not properly inflated at reductions of the perfusion pressure (e g by partial clamping of the renal artery) In compensation the kidney might liberate angiotensin which by its effect on the systemic pressure again might raise the distending pressure It was suggested that there might be a pressor receptor as well as an effector endocrine gland in this circumscribed region containing a segment of the distal tubule and the end of the afferent arteriole According to Swann s hypothesis the distension of the interstitial space is responsible for the control of the release of angiotensin This concept is clearly incompatible with the observed fact that the proximal intratubular and peritubular capillary pressures are completely normal in most nephrons a few minutes after application even of

a rather tight partial clamp to the renal artery of rats (Leyssac 1964 b) In these kidneys the glomerular capillary pressures must have been significantly reduced (cf p p 85 and 89) further indicating that the intratubular pressure rather than the interstitial pressure is subject to regulation

Thurau (1964 b) advocates that the renin angiotensin system is involved in the renal autoregulation of blood flow and filtration supported by the observation by Waugh and Shanks (1960) who in isolated perfused kidneys found that the autoregulation was abolished when kidneys were perfused with colloid free solutions but recovered their autoregulation when plasma was added to the perfusion medium Thurau believes that the release of angio tensin is reduced as a consequence of reductions in the distal fluid sodium concentration at the site of macula densa This idea was based upon indi cations of low sodium concentration at this site, such as under conditions of osmotic diuresis in which vasodilatation occurs and on the finding by other investigators of an increased renin content in kidneys under conditions of sodium depletion reduced perfusion pressure to the kidneys obtained by clamping or by administration of pressor substances, and other conditions in which the sodium concentration at the macula site also must be reduced Consequently Thurau has to postulate first that liberation of angiotensin also is reduced under these conditions of increased renin content in the kidney, which is contrary to the direct evidence obtained (referred to above) Next he proposed that increased renal plasma flow at abrupt increases in renal perfusion pressure within the interval of autoregulation (80–100 mm Hg) initially would lead to increased distal tubular sodium concentration and flow

According to this hypothesis the increased sodium concentration would increase the liberation of angiotensin which again would reduce the plasma flow and filtration rate by an afferent vasoconstriction exclusively The author is of the opinion that the autoregulation and the glomerulo tubular balance is explained by this mechanism which opinion would be in accordance with the traditional interpretation of a proximal sodium reabsorption rate being load dependent Proximal sodium (– and fluid) reabsorption however must be described as a Tm limited process second Thurau postulates in this hypo thesis that endogenously released angiotensin were to act on the pre glome rular vessel an idea which does seem most unlikely At the site of the juxta glomerular apparatus the afferent arteriole has lost more or less its muscular media the plain muscle cells being replaced by the epithelioid cells (the JG cells) in which myofibrils always are absent or extremely scarce Any continuous circular muscle layer which may act as a sphincter is not seen at this site A continuous muscle ring is not found until at the site of the efferent

arteriole (Faarup personel communication) Thus the first site for a vascular action of angiotensin released in the kidney must be distal to the pre glomerular vessel (An action on the glomerular capillaries is also unlikely because of the absence here of plain muscle cells and, angiotensin has been found to be a typical constrictor of plain muscle cells (Folkow, Johansson and Mellander (1961))

Guyton Langston and Navar (1964) have also concerned themselves with the problem of renal autoregulation of the blood flow Several possibilities of feed back mechanisms at the juxtaglomerular apparatus have been taken into consideration and they have tested how predictions of these types of feed back according to the conventional concept of renal function fitted the data available in the literature finally they advocated the possibility of an osmotic feed back by which to control the afferent arteriolar resistance as the most likely type Based upon these considerations they presented a mathematical model of such osmotic feed back mechanism This model is based on the assumption that the post glomerular resistance is constant and the glomerular filtration rate proportional to the glomerular propulsive pressure and they postulated that the renal plasma flow varies directly with osmolality (at the site of macula densa) and with the arterial pressure Based on this model which thus involves untenable and unlikely assumptions they showed how computer predictions of the effect of varying different parameters of the model agree with known characteristics of renal function which finding they take as an indication that an osmotic feed back in the control of pre glomerular resistance could exist and be of significance to the autoregulatory phenomenon But they admit other types of feed back mechanisms should continuously be taken into consideration They believe that changes in the tubular fluid osmolality would change the contractility of the juxtaglomerular cells (by depolarization of the cell membrane) As discussed above these epithelioid cells may hardly be considered as contractile The idea that the renin angiotensin system might be involved in this auto regulating feed back mechanism was rejected because of the apparent conflict between the indications of vasodilatation during low arterial pressure to the kidney and vasoconstriction during conditions of sodium depletion both of which conditions are known to increase the release of angiotensin But to reject this idea merely on the basis of these arguments is hardly justified nor would it seem reasonable to consider mechanisms by which the intrarenal vascular resistances (and thus the blood flow) is regulated as a simplified system like the one proposed by Guyton here only one factor which acts solely on the pre glomerular arteriole is considered of significance Rather normally the regulation may be assumed to be a highly complex mechanism

and among other factors the afferent arteriole may be partly subject to nervous control in addition to its possible intrinsic myogenic, autoregulatory property, moreover the post glomerular tone may be controlled in part at least by an endogenous release of angiotensin The relative importance of these many factors to the actual renal blood flow and resistances in any particular experimental or physiological situation can hardly be predicted at present

It may be stated that the previous hypotheses on the function of the juxta glomerular apparatus are either inacceptable and/or insufficient as a means by which to correlate the likely stimulus to the effect elicited on the functional parameters of the kidney A general objection to all of these hypotheses besides the critizism already mentioned is that they are incompatible with or do not account for the significance of a proximal Tm for salt and water and the tubular effect of angiotensin by which changes in the glomerular filtration rate normally in all probability are mediated and by which the proximal intratubular pressure and the fluid flow to the distal segments must be significantly influenced

According to the hypothesis proposed by Leyssac (1964 b) any reduction in the proximal intratubular pressure whether caused by a reduction in filtration pressure (i e glomerular propulsive pressure) or by a primary increase in the rate of reabsorption in this segment will increase the release of angiotensin by some still unknown change at the site of macula densa resulting from the minor decrease in the proximal hydrostatic pressure (the release thus being related to the distal load of salt) The rate of proximal reabsorption will thereby be depressed due to the tubular action of angiotensin reducing the glomerular filtration rate, and the efferent arteriolar resistance will be increased due to its vascular action Both of these effects will support each other in restoring the proximal intratubular pressure towards normal levels and thus will counteract the angiotensin releasing stimulus

This hypothesis of a feed back mechanism operating across the juxta glomerular apparatus seems to be compatible with the existence of a proximal Tm limited fluid reabsorption process and with the two primary actions of *angiotensin It may* explain the regulation of the proximal intratubular pressure by which the fluid load to the distal tubular segments is maintained fairly stable and it is in accordance with the present evidence of a releasing stimulus to the renin angiotensin system It is a serious question however whether the results obtained in experiments on the renal autoregulation are compatible with this hypothetical mechanism which presumably operates under physiological conditions Although it is fully realized that this model can hardly be expected to explain all of the available data concerning the

subjects discussed in this review it still might be relevant to have the question discussed in this context If a feed back mechanism of the above suggested type under normal conditions operates across the juxtaglomerular apparatus it might actually be predicted to react in some way or other under experimental conditions by which the perfusion pressure to the kidney is changed Moreover indirect evidence of the participation of the renin angiotensin system in the phenomenon of autoregulation was gained when a plasma factor (possibly angiotensinogen) was found to be of importance to the autoregulation (Waugh and Shanks 1960) Further it has been demonstrated (e g Shipley and Study 1950 Thurau and Deetjen 1962) in semi isolated kidneys in situ (cf p 58) that the urine flow and salt excretion rate (osmolar clearance) increase almost exponentially when the renal perfusion pressure is elevated within the range 80 to 180 mm Hg in which range of pressure the glomerular filtration rates and renal plasma flows are maintained fairly constant In these experiments the increase in water excretion at the high pressure levels amounted to about 8–10 per cent of the filtered volume Taking into account the slowly variable and limited distal sodium Tm these data suggest (or at least do not exclude) a progressively diminishing proximal reabsorption rate during elevation of the perfusion pressure Consequently were the renin angiotensin system to be involved in autoregulation these data might suggest that the release of angiotensin would increase with increasing arterial perfusion pressure (and glomerular propulsive pressure) apparently in contrast to the present hypothesis and the evidence of an increased release under conditions of reduced renal arterial pressure and negative salt balance It should be emphasized however that such conditions by which the perfusion pressure to the isolated (denervated) kidneys is elevated by means of a pumping device is a rather extreme and unphysiological situation which hardly is comparable with normal conditions The two different experimental situations may be *complementary* each revealing one of two properties of the receptor system which cannot be observed together by the same procedure Considered from this point of view the attention should again be directed to the anatomical arrangement of the juxtaglomerular apparatus especially to one of the characteristics of the three dimensional models constructed by Barajas and Latta (1963) and Faarup (1964) (cf p 76) demonstrating the fact that the ascending limb of Henle first approaches the post glomerular arteriole of its affiliated glomerulus following it to the vascular pole of the glomerulus where it contacts the afferent vessel running along with this pre glomerular vessel throughout a certain length This characteristic counter current system between the distal tubule and the arterioles common for all nephrons is highly suggestive of the fact

that the arteriolar system in some way may be of significance also for the 'receptor mechanism at the juxtaglomerular apparatus possibly as a reference system against which stimulating changes in flow fluid composition or pressure in the distal lumen are percieved If this speculative concept may be accepted as a working hypothesis an increased output of angiotensin under conditions of increased perfusion pressure to the isolated (denervated) kidney may not be inexplicable nor contradictory Under such conditions the reference would be seriously changed possible to such a degree as to cause an apparently inversed response to an otherwise normal stimulus by which response the normal regulation of the intratubular pressure effectuated by angiotensin consequently would break down Actually the renal tissue pressure (representing the proximal intratubular pressure) is completely uncontrolled under such experimental condition and changes in parallel with the arterial perfusion pressure as shown by Hinshaw and his group (1964) (cf p 25) This situation is probably in direct opposition to more physiological conditions of elevated arterial pressure, imitated by induction of neurogenic hypertension, in which the elevated arterial pressure, in part at least, is caused by increased arteriolar resistance In this latter case the pressure or flow at the end of the afferent arteriole would probably be unchanged or even moderately reduced Al o a normal response from the juxtaglomerular apparatus and a normal pressure regulating effect of angiotensin should be anticipated Accordingly Thurau and Wober (1962) found the proximal as well as the distal intra tubular pressures to remain unchanged in rats which seem to have been under this experimental condition Thus the data obtained concerning auto regulation may seem to be fully compatible with this modification of the hypothesis suggested by Leyssac (1964 b) according to this modification the arteriolar system will act as a system for reference towards a normal angiotensin releasing signal acting from the luminal side of the distal tubule at the site of macula densa In this modification of the model a more complete functional correlation with the structural arrangement seems also to be obtained

Finally it should be mentioned that even though the antidiuretic hormone reduces the distal intratubular pressure when administered to water diuretic animals as shown first by Wirz (1956) which effect is known to be without influence on the proximal turnover rate of salt end renal plasma flow this fact does not necessarily invalidate the present interpretation and model (cf Sellwood and Verney 1954–55 referred to p 100) the possibility that the distal luminal pressure or distension may represent the adequate stimulus to the renin angiotensin system need not be invalidated although it provides a further evidence against such possibility The pos ibility is still open that these

two octapeptides (angiotensin and vasopressin) may have an antagonistic (competitive?) action on the proximal reabsorption process in mammals In fact some – although not conclusive – evidence of the enhancing effect of vasopressin on the proximal sodium transport in mammals has been presented by Clapp Watson and Berliner (1963) By micropunctures they showed that about 57 per cent of the filtrate had been reabsorbed at the midpoint of the proximal tubules in antidiuretic dogs as evaluated from a F/P inulin of 2 3 In water diuretic animals (3–4 hours after initiation of the diuresis) only 38 per cent (F/P inulin 1 6) of the filtered volume was reabsorbed at this site of the tubule In both conditions the proximal tubular fluid was isosmotic with dog plasma In similarly hydrated dogs infused with vasopressin (100 mU per kg of body weight per hour) the mean F/P inulin was 2 0 in the middle third of the proximal tubules (equal to a fractional reabsorption of 50 per cent) These ratios differed significantly Although the mean inulin clearances were claimed not to differ in the diuretic and non diuretic groups, possible changes in the absolute rate of reabsorption could however not be evaluated in these experiments as emphasized also by the authors because of the great variability in the clearance in dogs a rather small number of animals investigated and the abscense of individual control measurements before hydration nor was a possible vascular (preglomerular) action of the infused vasopressin excluded in these experiments in which measurements of plasma oncotic pressures and of possible changes in the proximal intratubular hydrostatic pressures also might have been of interest Along this line of argumentation attention should be directed to the fact that clearances of inulin were about 50 per cent lower than normal in rats with diabetes insipidus (Gertz, Kennedy and Ullrich 1964) In these (unloaded) rats the fractional reabsorption did not differ significantly from controls thus the proximal reabsorption rate had been depressed suggesting a relatively increased sensibility to endogenous angiotensin The question cannot be answered, however until the adequate stimulus to angiotensin and the possible influence of vasopressin action on this stimulus or on the effector sites has been clarified in detail These problems will therefore be important subjects for future research

In summary data forming the basis of the present review may be interpreted by the following hypothetical sequence of events A condition of a decreasing pre glomerular pressure (e g provoked by gradual obstruction of the renal artery) and an initial state of high intrarenal turnover rate of filtrate may be considered as an illustrating example By partial obstruction of the artery, thereby lowering the glomerular propulsive pressure the proximal intratubular pressure will decrease initially Hence some function of this decreased pressure at the site of the macula densa possibly amplified during

flow through the loop of Henle, will stimulate the release of angiotensin Consequently two effects will follow The proximal reabsorptive capacity for salt and water will be depressed and the post glomerular arteriole be constricted Both of these primary, intrarenal effects will tend to restore to normal the proximal intratubular pressure and the glomerular filtration rate will decrease Also the renal blood flow will be diminished because of the reduction in the pre glomerular pressure and the increase in the post glomerular resistance With a further reduction in the propulsive pressure the release of angiotensin will increase until a maximum effect on the proximal Tm is reached Even if more angiotensin were released with further obstruction of the renal artery the reabsorption rate would not be further depressed Consequently an increased fraction of the filtered volume is reabsorbed proximally and the load to the distal tubular segment will be diminished Hence the distal intratubular pressure will fall, as also demonstrated in such partially clamped kidneys (cf p 89), and the urine flow decrease (cf p 85) The reduced internal diameter of the distal tubule (equal to increased resistance to fluid flow) also observed under these conditions (Leyssac 1964 b) explains readily how the proximal intratubular pressure still can be maintained in spite of a decreased luminal flow rate If a further drop in the glomerular propulsive pressure occurs less fluid will finally be filtered than the proximal tubule is able to reabsorb and the luminal flow velocity will reach zero value upon which the proximal lumen occludes

It should be emphasized again *that the most essential resultant effect of this regulating mechanism is the fairly constant supply of salt to the distal part of the nephron maintained irrespective of such variations in the glomerular filtration rate as are provoked by stimuli to the highly reactive pre glomerular vascular system or by primary metabolic changes in the tubular transport capacity both of which may be unrelated to the salt balance*

In view of the present concept a number of other experimental data may be predicted or comprehended some of which shall be briefly discussed in the following chapter

CHAPTER VI

Some further observations relevant to the present hypothesis

a) *Hypothetical considerations on the location of the tubular action of angiotensin*

It has appeared from the previous chapters that the tubular action of angiotensin is a depression of the rate by which the proximal fluid volume is reabsorbed (a depression of a volume Tm) Consequently its action need not specifically be an inhibition of the sodium transfer rate (Tm^{P}_{Na}) but rather an inhibition of the Tm of all the predominant electrolytes and urea (cf p 67) Such action on the volume transfer rate without an appreciably changed composition of the proximal luminal fluid conforms closely with the concept that the tubular action of angiotensin serves to stabilize the proximal hydrostatic intratubular pressure in order to maintain a fairly stable supply of salt to the distal tubular segment Further according to the hypothesis previously proposed (Ch II) an inhibition of the rate of proximal fluid reab sorption would be equal to an inhibition of the normally rate limiting luminal micropinocytosis It might therefore be tempting to suggest that the tubular site of action of angiotensin were localized to the luminal brush border mem brane This suggestion is not inconsistent with the concept deduced from studies on pinocytosis in amoebae (e g Chapman Andresen 1962 Chapman Andresen and Holter 1964) according to which pinocytosis (which may be induced only by charged molecules as e g sodium chloride amino acids and proteins in solution) is induced by an initial adsorption of the inducer to the external mucopolysaccharide layer of the plasmalemma, which as a secondary event initiates channel formation The mammalian brush border is known to contain mucopolysaccharides as indicated by its staining reaction with periodic acid Schiff (PAS positive), and further that angiotensin is reversibly bound by heparin (a polysaccharide sulphuric ester) as shown by Jacques et al (1960) Thus it is not unlikely that angiotensin has a rather specific affinity to the luminal membrane Further it should be mentioned that angiotensin

even in very high concentrations has no influence at all on the oxygen consumption of incubated rat kidney cortical slices (Leyssac, Lassen and Thaysen unpublished), nor has it any effect on the enzyme activity of a Na K activated adenosin triphosphatase system isolated from mammalian kidney cortex (R L Post 1961 personel communication Bonting Canady and Hawkins 1964) Although these latter observations hardly lend any support to the suggestion they are not incompatible with the theory that the angiotensin action is localized to the luminal step in the transfer process

b) *Glomerular filtration rate and distal salt load*

One of the consequences of a regulation of the salt load or flow velocity to the distal tubular segments would be the well established lack of correlation between the intrarenal turnover rate of salt and the electrolyte excretion rates which is rather difficult to understand on the basis of the previous concept of a load dependent proximal reabsorption rate determined by the rate of filtration According to thi (latter) concept *any* increase in the filtration rate should result in a more abundant supply of sodium to the distal parts of the nephron (on the assumption of a constant fractional reabsorption proximally) If the reabsorptive capacity of the distal nephron segments is limited which seems to be a well established fact the rate of sodium excretion would vary as a certain nonlinear function of the filtration rate (cf Pitts 1963, p 191) Were this always the actual case the following observations are difficult to explain e g that administrations of growth hormone, repeatedly found to increase considerably the inulin clearance, induce sodium and potassium retention even in adrenalectomized animals (Stein et al 1952) According to the concept advocated here, hormonal or other metabolic stimulators primarily increasing tubular reabsorptive capacity enhance the glomerular filtration rate by their tubular effect lowering the proximal intra tubular (capsular) pressure This effect which will reduce the distal load of sodium is counteracted by an increased release of angiotensin by which the tubular fluid flow is largely maintained although not completely In conformity with this Thurau (1964 c) and associates showed that infusion of metabolites of adenosine triphosphate (ATP) as adenosine and adenylic acid which increase the metabolic activity and exert a vasodilating effect in other organs elicit an instantaneous reduction in the renal blood flow in dogs Thus *according to the present hypothesis the direction of the changes in the distal load which accompany increases in the glomerular filtration rate will depend on whether the primary cause of this increase is of vascular or tubular origin*

c) *The dilution diuresis and expansion of the extracellular fluid volume*

From a similar point of view the sequence of secondary events after rapid infusions of Ringer solutions as described by Bojesen (1954 b) (cf Ch III B a, p 59–60), may be interpreted in greater detail The immediate increase in the salt and water excretion rate following reductions in the oncotic pressure of the plasma (phase I), previously discussed in detail is explained most readily by the accompanying increase in the intratubular pressure before the proximal reabsorptive capacity has been changed The second phase – a measurably increased renal plasma flow proximal reabsorption rate and rate of filtration –, was always seen 10–15 min after such expansions of the blood volume whether or not changes in the oncotic pressure were provoked, probably this is a consequence of a depressed release of angiotensin induced some how by the effect of the elevated proximal tubular pressure which will follow reductions in the oncotic pressure and/or reductions in pre glomerular arteriolar resistance likely to occur as a response (neurogenic) from extrarenal volume receptors Moreover, the changed angiotensin output from the kidney influences assumedly the adrenal cortical secretion of mineralocorticoid hormones since it has been demonstrated repeatedly in recent years that angiotensin plays a dominant role in the physiological regulation of aldosterone secretion As a detailed discussion of the many data reported in the literature concerning this complex problem is beyond the scope of this review only a few of the established facts shall be mentioned

It has been demonstrated unequivocally that infusion of angiotensin increases the aldosterone excretion and secretion rate in man (Genest et al 1960 Laragh et al 1960 a) in the dog (Mulrow and Ganong 1961), and in sheep (Blair West et al 1962) also when injected directly into the adrenal artery this fact is now used as a test for the specificity of the angiotensin yield in the indirect renin assay In vitro stimulation by angiotensin of aldosterone production from adrenal cortical slices was shown by Kaplan and Bartter (1962) and Davis and his group has shown that the development of secondary aldosteronism following thoracic cava inferior constriction in hypophysectomized dogs was abolished by nephrectomy while removal of a series of other organs had no such effect (Davis et al 1961) Subsequent studies from these and other groups have confirmed the close interrelationship of angiotensin – and aldosterone plasma levels and salt balance (cf Ch V, B), several investigators consider angiotensin as the trophic hormone to aldosterone secretion (e g Laragh Cannon and Ames 1964 Davis 1964) But the problem is complicated by the demonstration first by Laragh and associates (1960) that the aldosterone secretion rate in spite of sodium depletion

uninfluenced in patients with depressed plasma potassium concentration On correction of the potassium loss by oral administration, the plasma potassium concentration rose abruptly as did simultaneously the aldosterone secretion rate Further evidence of the importance of potassium to aldosterone secretion was provided by Blair West and his group (Blair West et al 1962) who in their sheep preparation in which an adrenal gland is transplanted to the neck in order to infuse directly into the adrenal arterial supply and to collect adrenal venous blood showed that sodium loaded animals in contrast to un loaded animals did not respond to infusion of small doses of angiotensin with increases in the aldosterone and corticosterone secretion However, infusion of solutions containing a high potassium to sodium concentration ratio did elevate the aldosterone and corticosterone secretion in these loaded sheep without any appreciable effect on the cortisol secretion rate At the C I O M S meeting in Prague (aug 1963) R E Peterson reported also that admini stration of angiotensin to rats had failed to provoke an aldosterone stimula tion which fact is not explained by a high blind value of plasma aldosterone (great stimulation of endogenous angiotensin) due to the operative mani pulations since similar results were obtained in salt loaded rats in which the blind aldosterone plasma concentration was close to zero (Bojesen, personel communication) These observations emphasize that other (inhibitory or competitive) factors may become of great significance to an adequate re sponse of the adrenal cortex to stimulation by angiotensin This fact would seem reasonable and might actually be anticipated, since an invariable in crease in the aldosterone secretion rate caused by angiotensin even when released because of influences unrelated with the salt balance would result in salt retention whether the animals were in a state of negative salt balance or, as in the above experiments even were salt loaded

It still seems safe to state that angiotensin plays a major role as a physi ological stimulator of the aldosterone secretion At present this statement seems to be the most reasonable clue to an interpretation of the third phase in dilu tion diuresis secondary to expansions of the extracellular fluid space, viz a prolonged and moderate increase in the rate of salt excretion with increased sodium to potassium concentration ratio in the urine observed some 45–60 min after such expansions (Bojesen 1954 b) This observation is to be ex pected from the depressed release of angiotensin inferred from phase II by which the rate of aldosterone secretion would also be depressed Further evidence in favour of this interpretation was provided by Bojesen and Degn (1961) These authors demonstrated that plasma levels of aldosterone had increased markedly within 10 min in normal dogs after blood volume deple tion (bleeding) and again decreased markedly at a similar rapid rate after

volume expansion (transfusion) This observation might suggest indirectly that the third direct effect of angiotensin (the adrenal effect) is as rapid as its two intrarenal effects

d) *The renal effect of noradrenaline and other pressor substances*

In clearance studies on the effect of noradrenaline and of angiotensin on salt excretion it has repeatedly been shown that the renal response to these pressor substances is qualitatively similar The typical acute effect in human subjects and dogs is a reduction in urine flow sodium excretion rate and renal plasma flow with a more moderate reduction in the inulin clearance These effects have generally been ascribed solely to the vascular action of the substances which interpretation cannot be maintained since the Tm limited character of the proximal fluid reabsorption and the tubular effect of angiotensin on this Tm has been demonstrated directly Changes in the proximal reabsorption rate accompanying changes in renal plasma flow after infusions of noradrenaline therefore must be ascribed either to a rapid direct tubular action of noradrenaline or to a secondary effect mediated by a rapid intrarenal regulatory mechanism Since such direct and instantaneous effect of noradrenaline on the occlusion time in rats could not be demonstrated (Leyssac 1965 b) the changed renal tubular reabsorption rate was inferred to be a secondary event following the administration of noradrenaline In consistency with this inference Scornic and Paladini (1964) (cf p 95) observed significant increases in the plasma concentration of angiotensin following infusions into dogs of 2–4 μg of noradrenaline per kg of body weight per min Further it is a well established fact that a paradoxical response may be observed also in acute clearance experiments in dogs and human subjects receiving small amounts of noradrenaline (vide infra) As discussed by Kruhøffer (1960) a rise in systemic arterial pressure resulting from the profound effects of the substance also on other parts of the vascular system may counteract the decline in glomerular propulsive pressure produced by a predominant pre glomerular vasoconstriction The significance of this possibility has clearly been demonstrated by Langston et al (1962) They showed in areflex dogs (complete spinal anaesthesia) that the renal blood flow increased as a result of the increase in arterial pressure at infusion rates below 0 6 μg of noradrenaline per kg of body weight per min Above this infusion rate the renal effect of noradrenaline caused the blood flow to decrease notwithstanding the continous rise in arterial pressure The renal blood flow remained uninfluenced by infusions at the low rates if a constant renal arterial pressure was maintained by means of a controlled arterial constriction moreover when the

renal arterial pressure was not maintained constant but even increased renal blood flow would decrease at the high dosage levels Changes observed in creatine clearance and urine flow rate were seen to be parallel to those seen in the blood flow If noradrenaline was infused at a low rate the rise in glomerular filtration rate (10–15 ml/min) would be seen to exceed by far the rise observed in urine flow (1 0–1 5 ml/min) Thus, the rate of proximal reabsorption obviously increased during the infusion of small amounts of noradrenaline A rise in renal blood flow and propulsive pressure, as a result of infusion of small amounts of noradrenaline per unit of time would tend to increase the proximal intratubular pressure and thereby salt and water excretion rates According to the present hypothesis the result will be that less angiotensin will be released because of this increase in pressure thereby increasing the rate of reabsorption proximally, and vice versa during infusions of noradrenaline at rates above 0 6 μg per kg of BW per min Thus the finding reported by Langston and collaborators seems to fit the present hypothesis This hypothesis is also commensurate with the observation of a moderate reduction in the excretion rate of potassium and a simultaneous moderate increase in the excretion rate of sodium in response to prolonged but small rises in the blood concentration of noradrenaline provoked by intramuscular injections of the substance in oil (Duncan et al 1951, Bliss Rubin and Gilbert 1951) The significance of this finding which apparently represents the reverse of an aldosterone effect, has hitherto seemed obscure However, according to the above reasoning a decreased release of angiotensin should be predicted as a consequence of such small increases in the blood level of noradrenaline Since angiotensin seems to be of major importance to the regulation of aldosterone secretion (cf section b, of this chapter), a reduction in the secretion of aldosterone should represent a secondary event in a prolonged administration of small doses of noradrenaline

Similar arguments may be valid also in cases of other pressor substances the administration of any pressor substance in doses which will affect the glomerular propulsive pressure in some way or the other may be predicted to influence immediately the signal to the intrarenal control mechanism at the juxtaglomerular apparatus thus causing a change in the release of endogenous angiotensin

e) *The importance of a voluminous proximal fluid reabsorption in mammalian kidneys*

A functional characteristic of mammalian kidneys which has puzzled many physiologists is the apparent peculiarity that such voluminous amount

of plasma has to be filtered and reabsorbed again predominantly in the proximal segment of the nephron by an energetically expensive transport process in order to secure the excretion of an amount of final urine corresponding only to 1–2 per cent or less of the filtered volume However, having once realized that a prerequisite of a controlled excretion rate of salt seems to be a fairly constant load of salt to the distal segments determined by the hydrostatic pressure difference between the proximal and distal tubules (cf p 87–88) a rapid control mechanism by which a fairly stable proximal intra tubular pressure can be maintained seems to be of evident significance especially in homoithermic animals with a highly reactive vascular system Since such regulating mechanism hardly will be sufficiently stable and accurate if it were to be effectuated solely by an adjustment of the efferent arteriolar tone, an adjustment of the proximal reabsorption rate seemed to be required in order to provide to this pressure an additional stabilizing effect (cf p 90–91) which effect most likely is provided by the tubular action of angiotensin No doubt the efficiency of the regulating intrarenal mechanism will depend on the capacity of proximal fluid reabsorption and filtration Since the glomerular capillary hydrostatic pressure in mammals may vary considerably (probably over a range of some 5–10 mm Hg), a small capacity and thus range of variation of the proximal reabsorption relative to such change in the glomerular propulsive pressure would greatly limit the efficiency of the regulation On the other hand the great capacity and range of variation of the proximal reabsorption actually present in mammalian nephrons has opened the possibility of a highly effective pressure regulation and thereby of a stable and regulated salt excretion It is apparently in keeping with this concept of a regulation of the load of salt to the distal nephron segments that changes in this load serve as the adequate stimulus to the control mechanism

f) *The hypothesis considered in the light of evolution*

Finally it is in keeping also with the above mentioned concept that a juxtaglomerular apparatus containing the renin angiotensin system apparently is found only in homoithermic animals Bean (1942) reported that renin found in kidneys of several mammals and birds could not be detected in kidneys of poikilotherms (fish amphibia or reptiles) although these latter animals actually were seen to react with pressor response to renin extracted from mammalian kidneys It might be interesting to have the result of extensive studies on this subject, using one of the more sensitive and specific renin assays developed in recent years, any definite conclusions can hardly be drawn until such results are available. If the suggestion inferred from

Bean's report is found to be tenable it might be suggested that a renin containing juxtaglomerular apparatus had evolved concurrently with the loop of Henle and a warm blooded state which would seem meaningful in the light of the present hypothesis Water conservation and sodium conservation are equally essential in animals with a terrestial habitat The ability to make a urine which is more concentrated than the plasma which must have been part of the adaptation to terrestial life is seen only in birds and, to a much greater extent in mammals only because bird and mammal tubules are bent into the loops of Henle In birds most of the nephrons are of the reptilian type and only few have a loop In mammals all nephrons have a loop, the relative length of which is closely correlated with the concentrating ability of the integrated kidney (B Schmidt Nielsen and O Dell 1961) In mammals, which excrete their nitrogenous waste products as the osmotically active urea in contrast to the almost insoluble uric acid excreted by birds and reptiles a more efficient concentrating mechanism would also seem to be required in order to reduce the loss of water accompanying urea excretion especially in mammals adapted to an arid habitat Such desert mammals including desert rodents, the urine of which may achieve osmotic concentrations of 5 5–6 5 osm/l (cf K Schmidt Nielsen 1964 table XXVI) may also excrete an excess of water in a hypotonic urine when drinking water is available Similarly an efficient sodium conservating mechanism permitting also an excretion of excess of sodium and operating independent of the simultaneous water excretion would appear to be equally essential for the survival of mammals this requirement is further stressed in mammals in which regulation of the salt excretion is taken over entirely by the kidney (in contrast to the additional extrarenal salt excretion by salt glands seen in birds and reptiles), and which have no possibility of regulating the salt intake either by the oral or the percutaneous route Such efficient regulation would seem to be conditioned by a controlled salt load to the distal tubular segment in which the final regulation of the excretion rate takes place

In this context it should be mentioned also that a fairly stable tubular flow velocity in the loop of Henle is highly important for the concentrating ability of the counter current multiplier system in this structure The counter current hypothesis implies that the maximum concentration to be established by the system will be reduced by marked increases as well as decreases in the flow rate if flow rates are high because the concentration difference between the two limbs (which create the multiplication) will not be fully established on account of the insufficient time of contact with the fluid, in the case of very low flow rates because of back diffusion of water from neighbouring areas in which fluid is less concentrated According to Kuhn

(1962) a reduction in the flow rate in the loop by a factor of 10 would seriously impede the multiplication of osmotic concentration because of back diffusion However it should be emphasized again that it still remains an unanswered question to which extent the juxtaglomerular apparatus exerts a regulating influence on the proximal intratubular pressure in the juxta medullary nephrons which predominantly are engaged in the counter current system due to the long loops of Henle as opposed to the short loops and re latively long proximal convolutions of most nephrons in the outer cortex of many species In some species (certain desert rodents, Sperber 1944) in which all nephrons have long loops of Henle and for this reason probably contribute equally much to the counter current system and the salt excretion the re gulating effect of the renin angiotensin system on the intratubular pressure might probably be of importance also in the counter current system On the basis of our present knowledge however it is too early to enter into a more detailed discussion and interpretation of the integrated renal function

On the basis of the above reasoning it may be suggested tentatively that the juxtaglomerular apparatus is specifically evolved as a simple functional structural arrangement in the mammalian nephron concurrently with and interdependent with the loop of Henle thus providing a basis for a sodium conserving mechanism to operate in the distal segments as does the loop ar rangement for a water conserving mechanism also to operate in these seg ments

Final remarks

The purpose has been to review a few data, considered to be essential to the comprehension of the problem at issue the accomplishment and regulation of the proximal tubular reabsorption in the mammalian kidney, which problem is considered highly important if we are to understand the regulation of renal salt excretion and the extracellular fluid space The data presented have driven the author to dispute and reject some of the current assumptions and to propose instead alternative hypotheses with respect to the regulation of the reabsorption rate and the mechanism of transcellular transfer of the reabsorbate The impression may have arisen that the bulk of data might be comprised in a unifying concept, thus bringing into accord data obtained from studies on the integrated function of the whole kidney, on the single nephron and the proximal cell and on the transport characteristics of the individual constituents of the reabsorbate This functional concept seems largely to be in accordance with existing macroscopial and ultramicroscopical structural equivalents

It is my hope that the theory presented here, which only should be regarded as a rough scetch, may stimulate future research and conceptual approaches for future design of experiments which eventually may disclose new and additional physiological aspects Many of the details of and inferences from this hypothesis require a more exact formulation and further experimental verification, which no doubt will lead to modifications and further elaborations of the proposed concepts

Appendix Statistical analysis

by

Inger Harder Hansen

Statistical analysis

The present study concerns the relationship between the clearance of inulin, denoted by (v) and the reciprocal of occlusion time (u)

(v) has been measured 2–4 times in each rat hence it is possible to derive an estimate of the variance of (v) Contributors to this variance are changes in the rate of reabsorption during the collection periods and experimental errors When the mean value of (v) and the value of (v) in the last collection period, respectively, are plotted against (u) in each experiment the points are seen to scatter most in the latter case It therefore seems reasonable to use the means for a comparison with (u) Since repeated measurements of the occlusion time are not available, the variance of (u) cannot be estimated

In fig 1 (p 75) the mean of the measured values of (v), denoted itself by (v) is plotted against (u) in the group of control rats It seems reasonable to claim a linear relationship which may be derived by the method of linear regression analysis with (v) as independent variable, thus disregarding its variance The assumptions necessary for the application of this method are that the observations are independent and that (u) is normally distributed for each value of (v) with the same variance The constancy of the variance can roughly be cheked by grouping the observations according to (v) and computing the variance within each group Any tendency of the variance to decrease or to increase with (v) could not be demonstrated by this test and the values were reasonably constant When the assumptions regarding normality and constancy of variance and the linearity were further cheked they were found to be fulfilled in all cases

In the further analysis data (v) $>1\,60$ are disregarded since they are few only and scattered

The common estimate of the relationship between (u) and (v) for the control rats is $y=5\,82+4\,582\,(v-1\,091)$ with $s^2=0\,624$ $s\ =0\,0085$, and $s_b=0\,164$ each with 69 degrees of freedom A t test shows that this line does not differ significantly from the line through $(\bar{v}\ \bar{u})$ and $(0\,0)$ i e $y=5\,33$

(v) The relationship between (u) and (v) in control rats with (v) $\leqq 1\ 60$ may therefore be described by $(u) = 5\ 33\ (v)$ in which the estimate of the slope has the variance $s_b = 0\ 0071$ and $s = 0\ 670$, each with 69 degrees of freedom This line is given in all the figures

Dose response of angiotensin

The data concern the relationship between inulin clearance and occlusion time in rats given a do e of (d_) ng of synthetic angiotensin in the interval between the measurements of inulin clearance and occlusion time It is obvious from the data that angiotensin prolongs the occlusion time Hence the clearance of inulin is denoted by v(d) and the reciprocal of the occlusion time by u(d) (d) indicating the total dose of angiotensin (exogenous plus an endo genous equivalent) corresponding to its concentration in the renal peritubular plasma at the moment concerned

It was found above that the relationship between u(d) and v(d) might be described by $u(d) = \lambda\ v(d)$, with $\lambda = 5\ 33$ as an estimate of λ having $s^2_\lambda =$ 0 0071

On the assumption that the physiological variation in (u) is caused by an equivalent to endogenous angiotensin (d_1) $u(d_1+d_2)$ is plotted in fig 2, 3 and 4 against $v(d_1)$ for (d_2) equal to 25 ng 7 15 ng and 5 ng respectively $v(d_1)$ is represented by the mean of repeated measurements, assuming that (d_1) remains reasonably constant throughout the observation period The purpose is to describe a response curve $y = v(d)$ The function v(d) generates the curves around which the points in the fig 2–4 scatter, since it for any observed $v(d_1)$ determines (d_1) and for (d_1+d_2) determines $v(d_1+d\ )$ Hence $u(d_1+d_2) = \lambda\ v(d_1+d_2)$ giving a functional relationship between $v(d_1)$ and $u(d_1+d_2)$

Fig 2 suggests a dose response relationship with horizontal asymptote $y = \alpha$ giving

$$(1) \qquad v(d) = \beta\, e^{-\gamma d} + \alpha$$

in which α β and γ are unknown positive constants From this equation we obtain the following expression for $u(d_1+d_2)$ as a function of $v(d_1)$

$$(2) \qquad u(d_1+d\ ) = \lambda\, v(d_1+d\ ) = \lambda(\beta\, e^{-\gamma(d_1+d\ )} + \alpha =$$
$$\alpha\lambda\,(1 - e^{-\gamma d}\ ) + \lambda\, e^{-\gamma d_2} \times v(d_1)$$

An exponential dose response relationship of this form would yield straight lines with the slopes $(\lambda\, e^{-\gamma d}2)$ passing through $(\alpha,\ \alpha\lambda)$ But the registered

data do not fit strictly with straight lines for all the doses investigated and the estimate of (α) – (α) –, derived from the regression lines fitted to the observed data, was somewhat greater (0.949) at a dose of 5 ng than that estimated at the two other dose levels (0.805 and 0.836 respectively). This inconsistency emphasizes the fact that the dose response curve is not exactly the one expressed in equation (1). However, since the difference in the estimate of (α) is not very great this equation has been chosen as a first approximation to a quantitative description of the data. In order to find a usable approximation to a dose response curve of the form (1) $v(d)=\beta\, e^{-\gamma d}+\alpha$, we have to determine (α) and (γ) from the three straight lines shown in fig. 2–4 which agree reasonably well with the observed data. The best approximation seemed to be that $(\alpha)=0.83$ ml/min/g KW and $(\gamma)=0.2\ \mathrm{ng}^{-1}$ using these values the data are described by the regression lines given in the Table I (p. 79) and shown in figs. 2–4.

Although the estimates of (α) and (γ) are rough only the curve given by

(1a) $$v(d)=\beta\, e^{-0.2d}+0.83$$

seems to give a reasonable approximation to the dose response curve.

The parameter (β) cannot be estimated on the basis of the present data since the relationship between $u(d_1+d_2)$ and $v(d_1)$ does not include (β).

The reduction in glomerular filtration rate caused by administrations of (d_2) ng of exogenous angiotensin to a rat (250–300 g of body weight) at a state of function corresponding to the equivalent of (d_1) ng may be calculated as a percentage of the maximally possible reduction from the above dose response relationship as follows

(3) $$\frac{v(d_1)-v(d_1+d_2)}{v(d_1)-\alpha} = \frac{\beta\, e^{-\gamma d_1}+\alpha-(\beta\, e^{-\gamma(d_1+d_2)}+\alpha)}{(\beta\, e^{-\gamma d_1}+\alpha)-\alpha}=1-e^{-\gamma d}$$

Thus the exponential dose response function implies that the effect relative to the maximum response at any value of $(v\,(d_1))$ is independent of (d_1).

For $d_2=$ 5 ng the effect is $=1-e^{-0.2\times 5}$ $=63.2$ %

For $d_2=7.15$ ng the effect is $=1-e^{-0.2\times 7.15}=76.1$ %

For $d_2=$ 25 ng the effect is $=1-e^{-0.2\times 25}$ $=99.3$ %

Solving the above equation (3) with respect to (d_2) we get

(4) $$e^{-\gamma d_2}=\frac{v(d_1+d_2)-\alpha}{v(d_1)-\alpha}$$ and consequently

(5) $$d_2 = -\frac{1}{\gamma}\log_e \frac{v(d_1+d_2)-\alpha}{v(d_1)-\alpha} = -\frac{1}{0.2}\log_e \frac{v(d_1+d_2)-0.83}{v(d_1)-0.83}$$

Equation (5) expresses the amount of exogenous angiotensin in ng required to reduce the clearance of inulin in a rat kidney from $v(d_1)$ ml/min/g KW to $v(d_1+d_2)$ ml/min/g KW, using intravenous application.

In Table II (p. 82) (d_2) is calculated for a few values of $v(d_1)$ and $v(d_1+d_2)$.

Summary

The establishment of the general, biological requirement of salt balance in the organism, reflected in the maintenance of a stable volume of the extra cellular fluid space depends in mammals almost exclusively on a regulation of the renal salt excretion

If the mechanisms involved in such regulation of the renal excretion were to be disclosed and comprehended, a prerequisite would be that methods were developed which permitted a clear distinction between vascular and tubular factors in the renal handling of salt and water The review presented here is concerned mainly with data obtained from studies on the reabsorption of filtrate by the proximal tubules of mammalian nephrons in which tubular segment the great majority of filtered salt and water is reabsorbed This topic was chosen for the experimental investigation, on which this review is based because it was not generally agreed whether the rate of reabsorption of salt and water in this tubular segment is dependent or independent of the filtered load of salt The main theme of this review is the analysis of this problem and its implications which is of fundamental importance for the entire concept of the mechanisms involved in urine formation and especially for the understanding of the mechanisms involved in the regulation of the renal salt excretion

In the first chapter some early contributions to the understanding of the fundamental features in urine formation are briefly mentioned Originally the reabsorbed substances had been classified it appears in two groups those transferred because of passive back diffusion and the others – Tm substances – which are actively transferred by mechanisms of limited reabsorptive capacity The subsequent investigations and interpretations are discussed which apparently have side tracked this original concept and led to a generally accepted theory of the renal handling of salt and water It is emphasized that this theory is based on five interrelated assumptions which lack either direct experimental confirmation or have been disproved by direct observations Thus it has not been established by any direct evidence, that the clearance of inulin (GFR) is determined exclusively by glomerular vascular factors (Assumpt I) nor has it been directly demonstrated that the rate of proximal sodium reab

sorption is dependent on the filtered load per se (Assumpt II) An assumed rate limiting concentration gradient for sodium in the proximal tubules (Assumpt III) does not exist, and proximal urea reabsorption proceeds pari passu with the reabsorption of sodium and water and therefore cannot contribute to an establishment of such gradient (Assumpt IV) Finally it has been demonstrated indirectly as well as directly that the hydrostatic pressure in the space of Bowman s capsule is not a constant parameter (Assumpt V) but this pressure varies as a primary and immediate event parallel with changes in the glomerular propulsive pressure

The second chapter is concerned with transcellular transport processes it is attempted to characterize the reabsorption processes in the mammalian proximal tubules at the cellular level The generally accepted model for active transcellular sodium transport is outlined Gradient limitation and Tm limitation of active transfer processes in general are discussed and examples are given to show that active sodium transport across certain amphibian epithelia (frog skin and toad bladder) may be Tm limited Across these latter epithelia the entry of sodium into the cell from the morphological outside solution appears to be the rate limiting step in the transcellular transfer process Whether or not this step in the transfer also has a rate limiting effect on the reabsorption of salt in the proximal tubules of mammalian nephrons remains to be established A review is given of micropuncture studies, designed in order to characterize the transport of each individual of the predominant ion species of the ultrafiltrate across each of the two cell borders of the mammalian proximal tubular cell, viz the luminal and peritubular membrane The interpretation of these results according to the underlying model seems to be that sodium is not merely 'pumped actively out of the cell at the peritubular side it is also pumped into the lumen from the cell interior Also chloride ions apparently have to overcome a considerable electrochemical potential before they enter the cell at the luminal side and at this membrane potassium is actively pumped into the cell Other direct investigations are discussed in which the relative rates of proximal net transfer of the main constituents of the ultrafiltrate are compared Notwithstanding the differently directed and unequally sized electrochemical forces created by the active ion transports it has emerged that potassium, chloride bicarbonate and urea are reabsorbed along the convoluted segment together with sodium and water in the same proportion as the one seen in the luminal fluid moreover a change in the rate of sodium reabsorption is accompanied by a parallel change in the rate of transfer of all the other mentioned constituents Thus the stereotyped proximal fluid reabsorption is seen in main to have the character of a bulk

reabsorption of an aqueous solution of electrolytes and urea. Studies on the water permeability of the proximal tubular wall indicated that the permeability is too low to account for the actually occurring water reabsorption as being due to osmosis exclusively; finally, estimations of a very low intracellular sodium concentration throw serious doubt on the generally accepted concept that transcellular transferred sodium is pumped across the peritubular membrane from a homogenous cytoplasmatic sodium pool. On the basis of this analysis the transcellular transport in the mammalian proximal tubules is suggested to take place in two steps: a normally rate limiting luminal 'micropinocytosis' and a basal (peritubular) energy requiring mechanism of some yet undetermined, character. In this hypothesis the transcellular transport process is spatially separated from the active transport processes which are responsible for the maintenance of the cytoplasmatic ion composition. The kinetics of such hypothetical, transcellular transport mechanism and its bearing on the interpretation of certain experimental data are discussed. It is concluded that a characterization of the reabsorption rate of salt and water as a Tm limited reabsorption is quite compatible with the mechanisms of transcellular transport in mammalian proximal tubules, as outlined in the proposed hypothesis.

The third chapter is concerned with the crucial question whether the proximal reabsorption rate of salt and water is load dependent or Tm limited. Previously proposed models of load dependent mechanisms for proximal salt reabsorption are mentioned and criticized; it is emphasized that the measured data upon which the models are based, directly indicate that the reabsorption rate is of a Tm limited character although they have been interpreted differently by the authors. Data obtained by the indirect clearance method are reviewed: it is concluded that the three types of investigations (salt loadings, induction of mechanical and dilution diureses) all are compatible with and in the case of dilution diuresis strongly support the concept of Tm limitation of the proximal reabsorption rate. A direct method by which to estimate the proximal reabsorption rate, viz. the occlusion time method, which has been applied to solve the problem in question is discussed in some detail. The occlusion time is defined as the time interval between the interruption of the renal circulation and filtration until the proximal tubules are emptied because of completed reabsorption of the luminal fluid. It is concluded that the method is a usable although rather rough method for an estimation of the proximal reabsorption rate. Data obtained by this method have indicated directly that the proximal reabsorption rate is independent of the filtered load supplied to the cells locally as well as downwards along

the entire convoluted segment of the proximal tubule Thus, the data obtained by indirect as well as by direct methods have unequivocally demonstrated the Tm limited character of the proximal reabsorption rate of salt and water

The conclusion that the proximal reabsorption rate is Tm limited involves two main consequences which are discussed *in chapter IV*

First, hydrodynamic considerations on the pressure drop across the glomerular membrane and across the nephron will show that any change in the glomerular propulsive pressure (filtration pressure) will be accompanied primarily by a parallel change in the proximal intratubular pressure (and capsular pressure), which also is in accordance with indirect and direct evidence Any changes in the effective filtration pressure, and therewith in the filtration rate will not occur until the rate of proximal reabsorption has changed This fact actually means that the rate of filtration is determined by the rate of proximal reabsorption The implications of this realization for interpretations of clearance data are discussed It further implies some rigidity of the proximal tubular wall, which must be able to resist a certain pressure difference between the tubular lumen and the surroundings Otherwise any moderate changes in the intratubular pressure (and therewith in effective filtration pressure) induced by primary changes in the rate of reabsorption could not be established Findings, by which such rigidity of the tubular wall is demonstrated are discussed

Secondly, the fact that the proximal rate of reabsorption does vary within a physiological range indicates that the limiting Tm is variable and a factor had to be looked for which might be responsible for these changes Using the occlusion time method angiotensin which is released from the juxtaglomerular apparatus was found to have such direct tubular effect, by which the proximal reabsorption rate is immediately and reversibly inhibited Angiotensin was found to have a characteristic maximum tubular effect by which the reabsorption rate is depressed to the lowest physiological values On the basis of the dose response relationship a rough calculation will show that a change in the order of about 200 molecules per cell is sufficient to elicit a maximum physiological depression of the proximal reabsorption rate The localization of the renin angiotensin system to the juxtaglomerular apparatus together with the vascular and tubular actions of angiotensin which readily explain the close correlation between and physiological range of variation in the renal blood flow the proximal reabsorption rate and the rate of glomerular filtration form the basis on which the conclusion is drawn that angiotensin is a physiological regulator of these parameters Since the maximum tubular effect of angiotensin can be responsible for a depression of the

rate of filtration only to the lowest physiological values, other mechanisms are required by which to explain unphysiological (pathological or experimentally provoked) reductions in the clearance of inulin In this survey data are included which demonstrate that such abnormal reductions in the inulin clearance are caused by the proximal luminal occlusion of ever increasing numbers of nephrons thus reducing the number of nephrons which participate in the urine formation

In the fifth chapter conditions for a regulated salt excretion of the mammalian kidney is discussed in the light of the regulated and quickly variable Tm limitation of the proximal reabsorption rate It is argued that such regulated salt excretion rate, which is extremely critical for the maintenance of salt balance, and which might be seriously impaired by occurring vascular and metabolic changes unrelated with salt balance is conditioned by an efficient regulation of the proximal intratubular pressure which will maintain a fairly constant load of salt to the distal tubular segments Data are reviewed which indicate that this pressure actually seems to be efficiently stabilized by some mechanism the possibility is analysed that changes in the release of angiotensin may be responsible for this regulation It is apparent from the literature that the release of renin or angiotensin generally is stimulated under conditions of decreased glomerular propulsive pressure probably by a stimulus acting from the tubular luminal side at the site of macula densa Data obtained from one extreme experimental situation in which the arterial perfusion pressure to the isolated kidney is artificially elevated suggest indirectly however that the release of angiotensin may be increased also in this situation The inference from such experiments is tentatively considered to suggest that the arteriolar vascular system may serve as a system of reference for normal stimuli to the juxtaglomerular apparatus acting from the tubular side After a critical review of the previous hypotheses on the function of the juxtaglomerular apparatus the available data are comprised in a hypothesis viz that the physiological function of the juxtaglomerular apparatus is to establish a rapid intrarenal feed back mechanism whereby some function of a change in the proximal intratubular pressure controls the release of angiotensin thus involving that the proximal reabsorptive capacity and the efferent arteriolar resistance are adjusted in such a way that a stable proximal intratubular pressure is maintained A resultant effect of this regulating mechanism is the maintenance of a fairly constant load of salt to the distal tubular segments irrespective of variations in the intrarenal turnover rate of salt and water provoked by stimuli unrelated to salt balance

In the last chapter (VI) a few hitherto obscure, observations are discussed which may be explained in the light of the proposed thesis Thus in view of the hypothesis that a luminal micropinocytosis represents the rate limiting step in proximal fluid transfer angiotensin might be presumed to have a specific affinity to this mucopolysaccharide containing membrane this suggestion is not contradicted by the demonstrated, reversible affinity of angiotensin to heparin (a sulphuric ester of a polysaccharide) The lack of correlation between the glomerular filtration rate and the electrolyte excretion rates under various conditions is discussed and on the basis of the hypothesis advocated here it might be predicted but would seem unexplainable if considered on the basis of the previous concepts Changes in the rates of electrolyte excretion renal blood flow and filtration rate induced by expansion of the extracellular volume are explained in accordance with the thesis and the well established stimulating effect of angiotensin on the adrenal aldosterone secretion The qualitative similarity of the integrated renal functional responses to heavy doses of angiotensin and noradrenaline irrespective of the abscence of any direct immediate tubular effect of noradrenaline is discussed according to the hypothesis this and other features observed concerning the renal effects of noradrenaline may be explained as secondary events mediated by rapid changes in the release of endogenous angiotensin The necessity of a voluminous proximal fluid reabsorption characteristic of all mammalian nephrons is comprehensible when it is realized that a stable proximal intratubular pressure is a prerequisite of a regulated salt excretion rate controlled by a mechanism which has to operate not only by changing the post glomerular vascular resistance but also the proximal reabsorptive capacity With a great capacity and range of variation relative to occurring changes in the glomerular propulsive pressure the possibility for an efficient pressure regulation has been created It is mentioned finally that the proposed regulating mechanism permitting an efficient sodium conservation to operate independent of simultaneous water excretion seems equally necessary for homoithermic urea excreting animals adapted to terrestial life as does an efficient water conserving mechanism Thus it is in accordance with the present thesis that the juxtaglomerular apparatus containing the renin angiotensin system to our knowledge is found only in birds and mammals and thus seems to have been evolved concurrently with and interdependent with the loop of Henle

Dansk resume

Det generelle biologiske krav om saltbalance i organismen, som finder udtryk i existensen af et stabilt extracellulært vædskerum, kan for pattedyrs vedkommende kun opfyldes ved en regulering af den renale saltudskillelse. For at kunne afsløre og forstå de mekanismer, der betinger en sådan regulering af nyrens saltudskillelse, har det været nødvendigt at udarbejde metoder, som tillod en klar adskillelse mellem vasculære og tubulære faktorers indflydelse på nyrens behandling af salt og vand. Denne oversigt beskæftiger sig overvejende med data opnået i studier over filtratreabsorptionen i pattedyr nefronernes proximale tubuli, hvor langt den største del af det filtrerede salt og vand reabsorberes. Dette emne for den eksperimentelle del af undersøgelsen, hvorpå oversigten er baseret, blev valgt, fordi der ikke var almindelig enighed om, hvorvidt reabsorptionshastigheden for salt og vand i dette tubulus afsnit er afhængig eller uafhængig af det filtrerede salttilbud. Denne oversigts afhandlings hovedtema er analysen af dette problemkompleks, som har fundamental betydning for hele vor opfattelse af de mekanismer, der ligger til grund for urindannelsen, og især for de, der betinger reguleringen af saltudskillelsen.

I første kapitel omtales kort nogle tidligere bidrag til forståelsen af hovedtrækkene i nyrens funktion. De reabsorberede stoffer synes oprindeligt at være blevet klassificeret i to grupper: Stoffer, der transporteres ved passiv tilbage diffusion, og de andre – Tm stoffer – der aktivt transporteres af mekanismer med begrænset transportkapacitet. De senere undersøgelser og tolkninger som øjensynligt har afsporet denne oprindelige opfattelse og ført til en generelt antaget teori for nyrens behandling af salt og vand, bliver diskuteret. Det fremhæves, at denne teori bygger på fem indbyrdes afhængige antagelser, som enten mangler direkte eksperimentel støtte eller er modbevist af direkte iagttagelser. Det er således aldrig blevet vist direkte, at inulin clearance (= GFR) udelukkende er bestemt af de vasculære faktorer i glomeruli (I. antagelse), ligesom det heller aldrig er blevet påvist direkte, at reabsorptionshastigheden for natrium er afhængig af selve tilbudet (II. antagelse). Det er vist direkte, at der ikke eksisterer nogen hastighedsbegrænsende concentrationsgradient for natrium i de proximale tubuli (III. antagelse), og at urinstof reabsorptionen her går parallelt med vand- og saltresorptionen og derfor ikke kan bidrage

til tilvejebringelse af en sådan postuleret natrium gradient (IV' antagelse) Endelig er det blevet vist både indirekte og direkte at det hydrostatiske tryk i Bowman s kapselrum ikke er en konstant parameter (V' antagelse), men tværtimod primært og umiddelbart varierer parallelt med ændringer i det glomerulære propulsive tryk

Det andet kapitel omhandler transcellulære transportprocesser da formålet har været et forsøg på at karakterisere reabsorptionsprocesserne i pattedyrnyrens proximale tubuli på det cellulære niveau Den generelt antagne model for transcellulær natriumtransport skitseres Gradient limitation og Tm limitation for aktiv transport i almindelighed diskuteres og der gives eksempler på at aktiv natriumtransport over visse amphibieepithelier (froskind og tudseblære) kan være Tm limiteret I disse epithelier synes det at være natriums optagelse i cellen fra opløsningen på dens morfologiske udside, der er det limiterende trin for den transcellulære transportproces hastighed Det vides ikke om det også er dette trin, der er hastighedsbegrænsende for saltreabsorptionen i pattedyrnefronernes proximale tubuli Der gøres rede for resultaterne af en række micropunkturundersøgelser, der har taget sigte på at karakterisere transporterne af natrium og ultrafiltratets andre ioner over hver af de to cellegrænser i pattedyrnyrers proximale tubulusceller, henholdsvis den luminale og den peritubulære membran Ud fra den tilgrundliggende model opfattelse må disse resultater fortolkes således at natrium ikke alene pumpes aktivt ud af cellen over den peritubulære membran, men også pumpe ind i lumen fra cellen Klor ionerne må tilsyneladende også overvinde et betydeligt elektrokemisk potentiale for at komme ind i cellen fra lumenvædsken og over den luminale membran pumpes kalium aktivt ind i cellen Der omtales en række andre undersøgelser som tillader en sammenligning af de relative netto transporthastigheder i de proximale tubuli af ultrafiltratets hovedbestanddele Det har vist sig at kalium, klor bicarbonat og urinstof reabsorberes sammen med natrium og vand ned igennem det proximale slyngede segment i det samme forhold som findes i tubulusvædsken til trods for de forskelligt rettede og uens store elektrokemiske kræfter, der er skabt af de aktive ion transporter og ydermere at en ændring i natrium reabsorptionshastigheden vil ledsages af en parallel ændring i transporthastigheden af alle de nævnte bestanddele af filtratet Den noget stereotype proximale væd kereabsorption fremtræder således i det væsentlige som en bulk reabsorption af en vandig opløsning af electrolyter og urinstof Undersøgelser over den proximale tubulusvægs vand permeabilitet har vist at permeabiliteten er for lille til at kunne forklare den faktisk forekommende vandreabsorption som ren osmose og endelig har det været muligt at skønne at den intracellulære natrium concentration er så

ringe at det i høj grad må betvivles at transcellulært transporteret natrium *kan pumpes over den peritubulære membran fra en homogen cytoplasmatisk* natrium pool

På basis af denne analyse fremsættes hypothesen, at den transcellulære transport i pattedyrnyrens proximale tubuli sker i to trin En under normale forhold hastighedsbegrænsende luminal micropinocytose ' og en basal (peri tubulær), energikrævende mekanisme af en eller anden art Ud fra denne hypothese er den transcellulære transportproces rumligt adskilt fra de aktive transportprocesser, der opretholder cytoplasmaets normale ion sammensæt ning Kinetiken for en sådan hypothetisk transcellulær transportmekanisme og dens relation til tolkningen af visse forsøgsresultater bliver diskuteret. Det konkluderes at muligheden for Tm limitering af hastigheden for salt og vandreabsorptionen i pattedyrenes proximale tubuli er helt forenelig med op fattelsen af den transcellulære transportmekanisme således som den er skit seret i den fremsatte hypothese Endelig gives der en kort omtale af den proximale glukosereabsorption set i lyset af denne hypothese

Det tredie kapitel omhandler det afgørende spørgsmål om hvorvidt den proximale reabsorptionshastighed for salt og vand er , load afhængig eller Tm limiteret Tidligere fremsatte modeller til forklaring af loadafhængige mekanismer for den proximale saltreabsorption nævnes og kritiseres og det fremhæves at målingerne på hvilke modellerne er baseret, faktisk direkte viser, at reabsorptionshastigheden må være Tm limiteret, skønt forfatterne tolker resultaterne anderledes Der gennemgås resultater opnået med den indirekte clearance metode og det sluttes at de tre forsøgstyper (salt belast ning mekaniske diureser og fortyndingsdiurese) alle har givet resultater der er forenelige med og for fortyndingsdiuresens vedkommende stærk støtter opfattelsen af at den proximale reabsorptionshastighed er Tm limiteret En direkte metode til vurdering af den proximale reabsorptionshastighed occlu sionstids metoden som har været brugt til at løse det pågældende problem diskuteres indgående Occlusionstiden er defineret ved den tid der medgår fra afbrydelse af nyrens kredsløb og filtration indtil de proximale tubuli har tømt sig for luminal vædske ved den fortsatte reabsorption Det sluttes at denne metode er en anvendelig omend temmelig grov metode til vurdering af den proximale reabsorptionshastighed Resultater opnået med denne metode har direkte vist at den proximale reabsorptionshastighed er uafhængig af det fil trerede tilbud til cellerne lokalt såvel som ned langs hele det slyngede *segment* af den proximale tubulus Således har målingerne både med indirekte og di rekte metoder utvetydigt vist den Tm limiterede karakter af den proximale reabsorptionshastighed for salt og vand

De to væsentligste konsekvenser af konklusionen At den proximale reabsorptionshastighed er Tm limiteret – diskuteres i *fjerde kapitel*

For det første vil hydrodynamiske overvejelser over trykfaldene over glomerulusmembranen og over nefronet vise, at enhver ændring i det propulsive tryk (filtrationstrykket) primært vil ledsages af en parallel ændring i det proximale intratubulære tryk (og kapseltrykket) i overensstemmelse med indirekte og direkte opnaede maleresultater Der vil saledes ikke forekomme ændringer i det effektive filtrationstryk og dermed i filtrationshastigheden førend den proximale reabsorptionshastighed ændres hvilket faktisk betyder at filtrations hastigheden er bestemt af hastigheden af den proximale reabsorption Betydningen af denne erkendelse for tolkningen af clearance resultater bliver diskuteret Det indebærer yderligere at der ma være en vis stivhed af den proximale tubulusvæg, som ma være i stand til at modsta nogen trykforskel mellem lumen og omgivelserne I modsat fald ville selv beskedne ændringer i det intratubulære tryk (og dermed i det effektive filtrationstryk) fremkaldt ved primære ændringer i reabsorptionshastigheden ikke kunne opsta Der omtales malinger der paviser en sadan stivhed af tubulusvæggen

For det andet viser den kendsgerning at den proximale reabsorptionshastighed varierer inden for et fysiologisk omrade at den hastighedsbegrænsende Tm er variabel hvorfor man matte søge efter en faktor der kunne tænkes at være ansvarlig for de forekommende ændringer i den proximale reabsorptions kapacitet inden for dette omrade Det er blevet vist med occlusionstids metoden at angiotensin der frigøres fra det juxtaglomerulære apparat har en sadan direkte tubulær virkning hvorved den proximale reabsorptionshastighed hæmmes øjeblikkeligt og reversibelt Der er pavist en karakteristisk maximal tubulær angiotensineffekt hvorved reabsorptionshastigheden bliver reduceret til den laveste værdi der ses spontant Et groft skon baseret pa dosis response afhængigheden viser at en ændring pa omkring et par hundrede molekyler angiotensin per celle er i stand til at udløse en maximal fysiologisk hæmning af den proximale reabsorptionshastighed Lokaliseringen af renin angiotensin systemet til det juxtaglomerulære apparat og den vasculære og tubulære virkning af angiotensin som utvungent forklarer den nøje korrelation imellem og det fysiologiske variationsomrade af nyrens gennemblødning den proximale reabsorptionshastighed og filtrationshastigheden er de præmisser der fører til konklusionen at angiotensin er en fysiologisk regulator af disse parametre Da den maximale tubulære angiotensineffekt kun kan være ansvarlig for et fald i filtrationshastigheden ned til det laveste omrade der ses fysiologisk ma der være andre mekanismer der er ansvarlige for ufysiologiske (pathologiske eller experimentelt fremkaldte) fald i inulin clearance Der omtales undersogelser der har vist at sadanne abnorme fald i inulin clearance skyldes occlu

sion af proximale lumina af flere og flere nefroner hvorved antallet af nefroner der deltager i urindannelsen aftager

I femte kapitel diskuteres forudsætningerne for at have en reguleret saltudskillelse i pattedyrnyren set udfra den situation at der er en reguleret og hastigt variabel Tm limiteret proximal reabsorptionshastighed Det pointeres at betingelsen for en sadan reguleret saltudskillelseshastighed som ma være overordentlig kritisk for opretholdelsen af saltbalancen og som i hoj grad vil kunne trues af forekommende vasculære og metaboliske ændringer der i sig selv ikke har nogen relation til saltomsætningen ma være en effektiv regulering af det proximale intratubulære tryk hvorved et ret konstant salttilbud til de distale tubuafsnit vil kunne opretholdes Der gennemgas resultater som viser at dette tryk faktisk forekommer at være effektivt stabiliseret af en endnu ukendt mekanisme og den mulighed at ændringer i angiotensinfrigørelsen kunne være ansvarlig for denne regulering analyseres Det fremgar af litteraturen at renin eller angiotensinfrigørelsen sædvanligvis stimuleres under forhold hvor det glomerulære propulsive tryk tenderer til at falde sandsynligvis forarsaget af et stimulus der virker fra den tubulært luminale side ud for macula densa Imidlertid lader resultater opnaet i en extrem experimentel situation hvor det arterielle perfusionstryk til den isolerede nyre kunstigt hæves indirekte formode at angiotensinfrigørelsen ogsa i denne situation kan være øget En sadan formodet frigørelse i denne situation kan muligvis tages som udtryk for at arteriolerne tjener som et referencesystem for normale stimuli til det juxtaglomerulære apparat fra tubulus-siden Efter en kritisk gennemgang af tidligere hypoteser om det juxtaglomerulære apparats funktion forsoger man at indordne tilgængelige data i følgende hypothese At det juxtaglomerulære apparats fysiologiske funktion er at etablere en feed back mekanisme hvorved visse ændringer der opstar som en ikke nøjere defineret funktion af ændringer i det proximale intratubulære tryk regulerer angiotensinfrigørelsen saledes at den proximale reabsorptionskapacitet og den efferente arteriolemodstand tilpasses efter forholdene pa en sadan made at det proximale intratubulære tryk holdes stabilt En sadan reguleringsmekanismes effekt vil være at vedligeholde et temmelig konstant salttilbud til de distale tubulusafsnit uafhængig af ændringer i den intrarenale omsætningshastighed af salt og vand der matte være fremkaldt af stimuli der er uden relation til organismens saltbalance

I det sidste kapitel (VI) forsøges nogle enkelte tidligere uforklarlige iagttagelser forklaret ud fra den fremsatte these Ud fra den opfattelse at det hastighedsbegrænsende trin i den proximale vædsketransport er en luminal

micropinocytose skulle man forvente en ret specifik affinitet af angiotensin til den luminale membran der indeholder betydelige mængder mucopoly saccharid, en formodning der kunne passe med en påvist reversibel affinitet af angiotensin til heparin (en svovlsyreester af et polysaccharid) Den mang lende sammenhæng mellem filtrationshastigheden og electrolyt udskillelses hastigheden under en række tilstande er forudsigelig ud fra den fremsatte hypothese men uforstaelig i lyset af tidligere opfattelser De ændringer, der fremkommer i electrolytudskillelseshastighederne nyrens gennemblødning og i filtrationshastigheden ved expansion af det extracellulære vædskerum, forkla res i overensstemmelse med thesen og det fastslaede forhold, at angiotensin stimulerer binyrernes aldosteronsekretion Den kvalitative lighed imellem de integrerede nyrefunktionsændringer der fremkommer under indflydelse af kraftigt virkende doser angiotensin og noradrenalin pa trods af den kendsger ning at noradrenalin ikke har nogen øjeblikkelig direkte tubulær virkning bli ver omtalt, og denne og andre iagttagelser over noradrenalins renale virknin ger forklares i overensstemmelse med hypothesen som secundære effekter udløst ved hurtige ændringer i frigørelsen af endogent angiotensin Nar det erkendes, at en forudsætning for en reguleret saltudskillelse er et stabilt proxi malt, intratubulært tryk der kontrolleres af en mekanisme som nødvendigvis ma virke ikke blot ved ændringer i den post glomerulære karmodstand, men ogsa i høj grad ved ændringer i den proximale reabsorptionskapacitet, bliver nødvendigheden af en sa voluminøs proximal vædskereabsorption som er ka rakteristisk for alle pattedyrnefroner ogsa forstaelig Med stor kapacitet og et stort variationsomrade i forhold til de muligt forekommende ændringer i det glomerulære propulsive tryk er der skabt muligheder for en effektiv trykregu lering Til sidst nævnes det at en sadan reguleringsmekanisme der gør det muligt for organismen at konservere natrium effektivt og uafhængigt af sam tidig vandudskillelse ma være lige sa nødvendig som en effektiv vandkonser verende mekanisme for varmblodige, urinstofudskillende dyr, der er adapteret til et liv pa landjorden Det synes derfor at være foreneligt med den fremsatte these at et juxtaglomerulært apparat med renin angiotensin systemet, savidt vides kun forekommer hos fugle og pattedyr hvor det saledes synes udviklet i indbyrdes afhængighed af og samtidigt med udviklingen af Henle s slynge

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Subject index

Numbers refer to pages of the present review in which the subject is discussed letters (A–H) refer to previously published papers in which the subject is dicussed Previously published papers are enlisted in front of this review

ACTA PHYSIOLOGICA SCANDINAVICA
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CIRCULATORY AND RESPIRATORY ADAPTATION DURING PROLONGED EXERCISE

BY
LARS-GORAN EKELUND

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CONTENTS

Preface

The present publication is based mainly on studies reported in the following papers

I Ekelund L G and A Holmgren Circulatory and respiratory adaptation during long term non steady state exercise, in the sitting position Acta physiol scand 1964 *62* 240—255

II Ekelund L G and A Holmgren Steady state diffusing capacity during prolonged, non steady state sitting exercise in ordinarily trained young men Acta physiol scand 1965 *65* 143—152

III Ekelund L G Circulatory and respiratory adaptation during prolonged exercise in the supine position Acta physiol scand 1966 *68* 382—396

IV Ekelund L G Circulatory and respiratory adaptation during prolonged exercise of moderate intensity in the sitting position Acta physiol scand 1967 In print

V Ekelund L G A Holmgren and C O Ovenfors Heart volume during prolonged exercise in the supine and sitting position Acta physiol scand 1967 In print

In the text these will be quoted as (I) and (II) etc

Introduction

The adaptation of the circulation and respiration during short periods of exercise of varying intensity has been extensively studied Recent reviews on this subject have been published by Asmussen & Nielsen (1955) Rushmer (1959) Wade & Bishop (1962) and Holmgren & Ekelund (1967) The studies have usually covered exercise periods of about 5—10 minutes which have been long enough to allow the organism to reach a relative steady state in respect of the different variables studied Steady state in a biological system is, however always a relative steady state i e the rate of change of some variable is within certain limits In short work tests a relative steady state in respect of heart rate is often defined as a change up to 10 beats/min between 2 and 6 min (Sjostrand 1960) Studies during prolonged continuous work for half an hour to several hours have been made by Krogh & Lindhard (1920) and Christensen & Hansen (1939) These were mainly metabolic studies with investigations of the respiratory quotients and blood glucose concentration during prolonged exercise and also of the thermo regulation Ekelund & Holmgren (1962) gave in a preliminary report data from heart catheterization studies in six healthy men during prolonged sitting exercise Cobb & Johnson (1963) measured the cardiac output during prolonged exercise by the dye-dilution method and also studied the free fatty acid metabolism In 1964 Saltin & Stenberg pub lished a report on prolonged severe exercise which was performed both on a bicycle and on a treadmill with short intermittent rest periods Dye dilution cardiac output and the brachial artery pressure were measured in four well-trained males From these earlier reports we know that there is often a non-steady state in respect of heart rate which is due to a fall in stroke volume

The principal aim of the present investigations was to study the variables of the central circulation such as cardiac output stroke volume heart rate and the pressures in the pulmonary and systemic circulation during prolonged exercise in a defined non steady state condition A simultaneous study of different respiratory variables was also of interest because there is often a non-steady state in respect of respiratory rate during prolonged exercise The same type of study was performed both in the sitting (I) and supine (III) position to investigate the influence of body position i e the influence of gravitation on the distribution of blood volume within the capacity vessels To investigate the importance of the relative intensity of exercise two studies were performed in the sitting position with different relative work loads (I IV) For a better

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CHAPTER I

Material and Methods

Material

The observations in the present report were obtained from studies in 29 healthy male volunteers of ordinary physical fitness All were blood donors registered at and controlled by the hospital blood bank The subjects were mainly students which explains the rather narrow age range (19—30 years) Three of the subjects took part in two investigations The anthropometric data are summarized in Table I and detailed information is given in each separate study

Comments The subjects were somewhat taller than the average for their age (mean 23 2 years) but in other respects comparable with for example military conscripts (Tornvall 1963) The low value for hemoglobin concentration is mainly due to the fact that the samples were taken when the subjects had rested supine for about 15 min which gives a value about 6 per cent lower than in sitting position (Ekelund & Kaijser 1966 unpublished observation) due to an increase in plasma volume

Table I Summarized anthropometric data from studies I—V The abbreviations are the same as in the text

	Age years	Height cm	Weight kg	Heart vol ml	THB g	THB g/kg b w	Hb conc. g/100 ml	Blood vol l	Blood vol ml/kg b w	W_{170} kpm/min sitting
$\bar{x}$	23 2	181 4	71 4	784	759	10.59	13 54	6 23	87 60	1 102
n	29	29	29	27	27	27	27	27	27	29
SD	2 5	6 8	6 7	106	97	0 87	0 75	0 83	6 37	165
range	19 30	167 197	58 86	645 985	560 985	8 2 12 2	12 1 14 7	4 9 8 6	74 3 100 0	750 1 500

Methods and Procedure

Methods

Detailed descriptions of methods and procedures have been reported in the separate papers and are therefore only briefly summarized below

Electrocardiograms were recorded with a direct writing ink jet recorder (Mingograf 42) both at rest in the supine and standing positions during exercise on a bicycle ergometer in sitting position and in the supine position after exercise using CR leads with modifications described by Holmgren & Strandell (1961)

Work capacity (W_{170}) was determined in the sitting and in 18 subjects also in the supine position by the method of Sjostrand (1960) on an electrically braked bicycle ergometer (Holmgren & Mattson 1954) with the work load increasing stepwise every sixth minute W_{170} is defined as the rate of work in kpm/min which a subject could perform at a heart rate of 170 beats/min The value of W_{170} was obtained by inter or extrapolation using the approximately linear relationship between pulse rate and work load

analysis of the findings from the heart catheterization, a study of the total heart volume determined by X ray was performed during prolonged exercise both in sitting and supine position (V) To further evaluate the respiratory adaptation during prolonged exercise another study was performed which incorporated measurements of steady state diffusing capacity during prolonged exercise in the sitting position (II)

and *base excess* were determined according to Andersen et al (1960) *Arterial oxygen tension* (P_O) was measured with a Clark microcathode electrode (polypropylene membrane) in a stainless steel cuvette kept at 37 C Measurements of pH P_{CO} and P_O were corrected for differences between body temperature and electrode temperature (Holmgren & McIllroy 1964

The mechanical efficiency was calculated from the equation

$$M = \frac{(\text{rate of work kpm/min}) \quad 100}{(\text{oxygen uptake l/min exercise} - \text{oxygen uptake l/min rest}) \quad 4\ 9 \quad 427}$$

Lactic acid concentration was determined in arterial blood by the colorimetric method of Barker & Summerson (1941) as modified by Strom (1949)

Mechanical systole The mechanical systole was measured from recordings of the brachial artery pressure with a paper speed of 100 mm/sec. Mean values were calculated from at least 3 usually 4—5 consecutive heart cycles The tip of the brachial artery catheter was usually introduced 3 dm in the central direct on

Body temperature The body temperature was measured as rectal temperature with a thermo couple (Ellab Copenhagen Typ TE 3) The rectal applicator was inserted to a depth of about 5 cm from the sphincter ani

Statistical calculations were mainly performed accord ng to Dahlberg (1940) and Snedecor (1959) Differences between regression lines were tested according to Hald (1960) The following probability (P) levels of significance were used $P < 0\ 001$ highly significant $0\ 001 < P < 0\ 01$ significant and $0\ 01 < P < 0\ 05$ probably significant

Procedure

The study started with determinations of some measures of the circulatory system such as total amount of hemoglobin total blood volume heart volume and rate of work at pulse rate 170 (W_{170}) A pilot test was also performed to select a work load which the subjects could sustain for about one hour in the decided body position The first four studies (I—IV) were performed in the morning after the subjects had had a l ght meal about one hour before the experimental procedure No sedative or quinidine was given The room temperature was between 20 and 24 5 C with a relative humidity of about 40 per cent The fluid losses blood and sweat were compensated for by continuous saline administration to keep the body weight constant (weight loss $< 0\ 7$ kg) Small amounts of water were also given per os Study V was performed lat r in the day about four hours after lunch In th s study no compensation was made for fluid losses No complications occurred during or after any of the experiments

Comments

All methods used during the different investigations were regularly checked with regard to validity and reproducibility The reproducibility calculated from duplicate estimations for every method agrees very well with the figures published for each separate method from this and other laborator es Table II shows the errors in W_{170} heart volume (prone) cardiac output and stroke volume lactic acid pH P_{CO} P_O and diffusion capacity of the lungs taken from Hellstrom & Holmgren (1966) Holmgren & Pernow (1960) Holmgren (1965 b) and Strandell (1964 b) The remaining values were calculated from measurements on the actual subjects plus ordinary subjects who took part in metabolic studies during the same period

All measurements with the exception of W_{170} heart volume (prone) oxygen up ake cardiac output stroke volume and diffusion capacity were made in duplicate

Table II Errors for a single determination as estimated from duplicate determinations

Variable	Experimental conditions	No of duplicate determinations	Mean value	Error $\pm\sqrt{\frac{\Sigma d^2}{2n}}$	Error in per cent of the mean value
W_{170} kpm/min	Sitting	56	1135	55 4	4 9
Tot Hb g	CO method semirecumbent	50	735	27 4	3 7
Blood vol l	CO method semirecumbent	50	5 43	0 239	4 4
Blood vol l	I^{131} albumin supine	49	5 04	0 224	4 4
Hemoglob conc g/100 ml	supine	40	13 77	0 095	0 69
Heart vol ml	prone	112	785	33 4	4 3
Heart vol ml	sitting exercise	44	777	38 1	4 9
Heart vol ml	supine exercise	34	888	36 7	4 1
V_O ml STPD/min	supine rest	35	289	10 6	3 7
V_O ml STPD/min	supine exerc sitt	35	1829	42	2 3
Oxygen saturat	exercise art	104	96 9	0 35	—
	exercise ven	103	41 8	0 34	—
Card output l/min	supine exercise	27	11 1	0 58	5 2
Stroke vol ml	supine exercise	26	92	6 3	6 8
Lact acid meq/l	arter blood	60	1 28	0 122	9 5
Lact acid meq/l	»	60	2 75	0 220	8 0
Lact acid meq/l	»	60	5 00	0 160	3 2
RQ	rest	35	0 773	0 031	3 9
RQ	exercise	35	0 818	0 025	3 1
P_{CO} mm Hg	Severinghaus	85	40 0	0 198	0 5
P_{CO} mm Hg	Astrup	39	37 0	0 93	2 5
pH units	Microelectr Astrup	50	7 383	2 2 10^{-3}	0 03
P_O mm Hg	Clark electr	100	77 5	0 93	1 2
$D_{L_{CO}}$ ml/min/mm Hg	sitting exercise	13	37 9	2 7	7 2
Mech syst c sec	Sitting rest + exer	50	23 7	0 43	1 8

CHAPTER II

Blood volume changes in connection with exercise

Previous investigations

Several earlier investigations (e g Thompson Thompson & Dailey 1928) have shown that plasma volume decreases on change of posture from supine to sitting and shortly after starting exercise both in supine and sitting position Before isotope methods became available two explanations had been proposed to account for this change the displacement of red cells from blood depots into the circulation or a concentration of the blood due to passage of fluid from the blood into the tissues Nylin (1947) found no change of the circulating red cell volume studied by isotope techniques in connection with exercise Uehlinger & Buhlmann (1961) studied the red cell volume during exercise by an isotope technique and at the same time the plasma volume with I^{131} albumin They injected chromium labelled erythrocytes however and labelled albumin only once before exercise and then measured the changes of concentration after estimation of the change of slope for I^{131} albumin They found an unchanged erythrocyte volume and a decrease in plasma volume by, on an average 10 per cent in sitting position and 3 6 per cent in supine position The exercise was of moderate absolute intensity (corresponding to an oxygen uptake 1 – 1 5 l/min) during 5 minutes Fricke (1965) studied the changes in red cell volume and plasma volume during exercise with isotope labelled erythrocytes and several injections of Evans blue The exercise was performed in the supine position and introduced no change in the red cell volume but there were significant changes in plasma volume with 5 to 20 per cent decrease from rest to exercise There was a correlation between the plasma volume decrease in per cent and the relative work load (expressed as per cent of W_{170}) The relation between total body hematocrit and peripheral blood hematocrit increased by on an average 3 2 per cent during exercise from 0 88 at rest to 0 90 during exercise On the assumption that changes in hemoglobin concentration reflected changes in total blood volume Holmgren (1956) estimated the decrease in blood volume during exercise and found already after 5 min exercise a decrease which after 15 min exercise amounted to 7 per cent of the resting value During prolonged continuous exercise of 30 min duration there was no further change in hemoglobin concentration Saltin & Stenberg (1964)

Table II Errors for a single determination as estimated from duplicate determinations

Variable	Experimental conditions	No of duplicate determinations	Mean value	Error $\pm\sqrt{\frac{\Sigma d^2}{2n}}$	Error in per cent of the mean value
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V_O ml STPD/min	supine exerc sitt	35	1829	42	2 3
Oxygen saturat %	exercise art	104	96 9	0 35	—
	ven	103	41 8	0 34	—
Card output l/min	supine exercise	27	11 1	0 58	5 2
Stroke vol ml	supine exercise	26	9?	6 3	6 8
Lact acid meq/l	arter blood	60	1 28	0 122	9 5
Lact acid meq/l	»	60	2 75	0 220	8 0
Lact acid meq/l	»	60	5 00	0 160	3 2
RQ	rest	35	0 773	0 031	3 9
RQ	exercise	35	0 818	0 025	3 1
P_{CO} mm Hg	Severinghaus	85	40 0	0 198	0 5
P_{CO} mm Hg	Astrup	39	37 0	0 93	2 5
pH units	Microelectr Astrup	50	7 383	2 2 10^{-3}	0 03
P_O mm Hg	Clark electr	100	77 5	0 93	1 2
D_{LCO} ml/min/mm Hg	sitting exercise	13	37 9	2 7	7 2
Mech syst. c sec	Sitting rest – exer	50	23 7	0 43	1 8

by Fricke (1965) who estimated red cell volume with radioactive chromium and plasma volume with Evans blue During the present investigations the I^{131} albumin was carefully checked for free iodine and denaturation of the albumin the presence of the latter would have given too high blood volumes especially during exercise In the first investigation samples were drawn from two of the subjects at different times after the injection to check the mixing time and that not too much of the albumin was lost from the intravasculary space The loss from the intravasculary space was found to be less than 2 per cent and therefore no correction was made The error which may be introduced if the elimination of albumin is more rapid during exercise than at rest is an underestimation of the blood volume decrease on start of exercise There is no reason why the elimination should change during exercise and as a consequence the values during exercise should be valid

CHAPTER III

Adaptation of the central circulation during prolonged exercise

Previous investigations

Cobb & Johnson (1963) published a study of some hemodynamic variables during prolonged exercise in a sedentary group of 14 males and in a physically active group of 7 males The subjects walked on a treadmill with the same load for both groups which gave an oxygen uptake of about 1 1 litre/min This corresponded to a high relative work load for the sedentary group, with a heart rate after 10 min of 176 beats/min The sedentary subjects could not walk for more than 15—36 minutes whereas the physically active group with a 10 min heart rate of 141 beats/min continued for at least one hour In both groups the dye dilution cardiac output was on an average constant over the whole work period, with small but insignificant changes Heart rate ro e progressively and significantly during exercise in all subjects with a corre sponding decrease in stroke volume In the sedentary group the average fall was 16 per cent from 4 to 6 min until the end of exercise In the physically active group the fall in stroke volume was 14 per cent from 4 to 6 min to the end of exercise (40—60 min) No pressures were reported Corresponding findings have been reported by Saltin & Stenberg (1964) who studied four well trained males during 180 min work both on treadmill and bicycle ergometer with an oxygen uptake of 75 per cent of the individuals maximal uptake, which corresponded to an oxygen uptake between 2 3 and 3 4 litre/min

In a study of 20 min treadmill exercise Levy, Tabakin & Hanson (1961) found that the cardiac output measured by dye dilution was maintained steady for 7 of the 12 subjects over the whole period and in all subjects between 8 and 20 min of exercise The oxygen uptake and ventilation were unchanged, but no values of heart rate stroke volume or pressures were given In another dye dilution study (Grimby Nilsson & Sanne 1966) 11 males exercised for 30 min in sitting position on a bicycle ergometer there were no significant changes in cardiac output but a small decrease in stroke volume corresponding to an in crease in heart rate

Present investigation

Cardiac output

In the 18 subjects studied with heart catheterization (I, III IV) in different body positions and at different relative intensities, cardiac output was meas

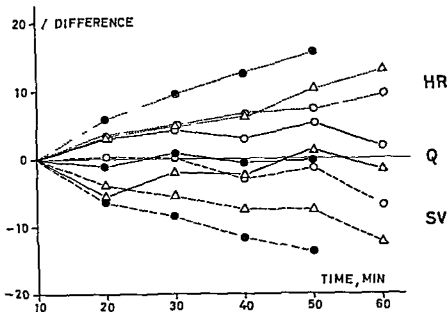


Fig 1 Response of heart rate HR ( ) cardiac output Q (———) and stroke volume SV ( ) during prolonged exercise mean values for six subjects in sitting study I (●) study IV (o) and in supine position study III (Δ) The data are presented as a percentage difference from the values obtained after 10 min exercise

ured every 10th minute during continuous work on a bicycle ergometer for 50—60 minutes There were no significant changes in cardiac output (Fig 1), but individual changes up to ± 1 litre/min the individual changes in cardiac output being followed by opposite changes in arterio venous oxygen difference The more or less marked increase in oxygen uptake (see chapter IV) was followed by a corresponding increase in arterio venous oxygen difference The change in cardiac output was not correlated to changes in respiratory rate total ventilation or body temperature The 10 min cardiac outputs fell well within the normal limits of cardiac output and oxygen uptake in the different body positions which have been published from this laboratory (Bevegard Holmgren & Jonsson 1960)

Heart rate

Heart rate increased continuously (measured for 1 minute every 10th min) in all studies both of circulation (I III IV) and of respiration and heart volume (II V) (Fig 1) The increase in heart rate was most marked in studies I and II

where the relative work load was about 77 per cent of W_{170} corresponding to a 10 min heart rate of 148 beats/min in study I. In studies with lower relative intensity (III, IV) there was also a significant increase in heart rate but not of as great magnitude as in the first study. The slope of heart rate with time was steeper in the first 30 min than during the last 30 min of exercise. These questions have been studied in detail by Ahlborg (1967). In the group of 18 subjects who took part in the heart catheterization studies there was a probably significant correlation between the increase in heart rate from 10 min to end of exercise and the relative work load either expressed as pulse rate after 10 min exercise or per cent of W_{170}: r = 0.56 for both. The increase in heart rate from 10 min to the end of exercise was in the present material highly significantly correlated to the increase in body temperature during the same period: r = 0.72. The heart rate during exercise was also significantly correlated to body temperature in 16 subjects with individual correlation coefficients between 0.87 and 1.00 (n = 5—6) and r = 0.64 for the whole group (n = 104). The increase in heart rate from 10 min to the end of exercise in the group of 18 subjects was not correlated to increase in total ventilation, orthostatic pulse increase, lactic acid concentration after 10 min exercise, decrease in total blood volume from rest to 10 min exercise, or increase in heart rate at a pulse rate of about 140 beats/min between 2 and 6 min exercise in the ordinary bicycle test.

Stroke volume

In all three circulatory studies (I, III, IV) there was a significant decrease in stroke volume (Fig. 1), the magnitude of which was related to the magnitude of the increase in heart rate during the prolonged exercise, for which reason the fall in stroke volume was most pronounced in the first study in sitting position at the highest relative work load. Between the two studies at the same relative work load in different body positions (III and IV) there was no difference in respect of stroke volume decrease. For the group of 18 subjects there was a *significant correlation between decrease in stroke volume between 10 min and end of exercise* ($\triangle$ SV $_{10\text{-end}}$) and the corresponding heart rate values ($\triangle$ HR $_{10\text{-end}}$):

$$\triangle SV_{10\text{-end}}\ (ml) = 1.66 + 0.663 \quad \triangle HR_{10\text{-end}} \quad r = 0.60 \quad S.D. = 7.98$$

The decrease in stroke volume shows the same relation to the other variables as the increase in heart rate mentioned above, so that the decrease in stroke volume is probably significantly related to the relative work load expressed as heart rate after 10 min work or per cent of W_{170}. The 10 min values fell within

the normal range of variation for the relationship between stroke volume during exercise and total amount of hemoglobin and heart volume (Holmgren, Jonsson & Sjostrand 1960 Bevegård et al 1960) in the different body positions

Intravascular pressures

In the three catheterization studies (I, III, IV) the pressures in the right ventricle, pulmonary artery and brachial artery were measured at rest and every 10th min during work The pulmonary wedge (PCV) pressure was recorded at rest in 16 subjects but at the beginning and end of exercise only in seven subjects On transition from rest to exercise all pressures rose to values which fell within normal limits in relation to cardiac output reported earlier from this laboratory (Holmgren & Ekelund 1967) During the continuous prolonged exercise there was a more or less continuous decrease of the pressures in the right ventricle, pulmonary artery and brachial artery (Fig 2) The pressure

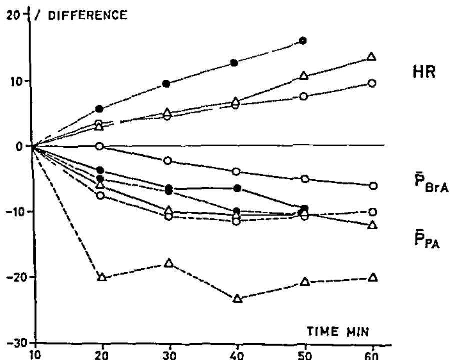


Fig 2 Response of heart rate HR ( ) brachial artery mean pressure $\bar{P}_{B A}$ (———) and pulmonary artery mean pressure $\bar{P}_{PA}$ ( ) during prolonged exercise For further data see fig 1

decrease in the brachial artery from 10 min to the end of exercise (50—60 min exercise) was of the order of 6—10 mm Hg with largest decrease in the group with the highest relative work load The magnitude and pattern of decrease in the mean pulmonary artery pressure was quite different in the two body positions In the sitting position, both at a high relative work load and at a medium work load, the decrease in pulmonary artery mean pressure was continuous and amounted maximally to 2 mm Hg at the end of exercise In the supine position on the other hand, the decrease was pronounced already between 10 and 20 min exercise (also observed by sancetta, 1957), after which it remained almost unchanged The maximal decrease was on an average 5 2 mm Hg and was mainly a result of a decrease in the diastolic pressure in the pulmonary artery The end diastolic pressure in the right ventricle decreased in all three studies by 2 2— 2 6 mm Hg during the continuous exercise There was the same magnitude and pattern of change in all three studies The PCV pressure showed no clear cut changes but should probably decrease as much as the diastolic pressure in the pulmonary artery because this is equal to the PCV pressure in normal subjects (Holmgren & Ekelund 1967) In supine position the PCV pressure was only recorded in two subjects during exercise and decreased in both In the sitting position at medium level of work intensity the PCV pressure was recorded in five subjects during exercise and was in these cases unchanged

Discussion

Cardiac output and metabolic requirements

In order to exercise continuously the working muscles have to receive a constant amount of oxygen per time unit plus the necessary fuel for combustion, such as free fatty acids and carbohydrates The two most important sources of energy are the circulating free fatty acids and local fat depots together with muscle glycogen (Havel et al 1964 a Ahlborg et al 1967) The relation between these two main sources varies according to nutritional state, relative work intensity and physical fitness The relation also changes during a work period as has been shown by RQ estimations during prolonged exercise (Christensen & Hansen 1939) In the present study the oxygen uptake remained steady or increased slightly when the work was of a high intensity The increase in oxygen uptake at a constant work load might partly be a result of a less efficient type of exercise in which more muscle groups are used at the end of exercise than at the beginning Another explanation could be that the combustion of fat requires somewhat more oxygen for each unit of energy compared with the combustion of carbohydrates about 10 per cent (Krogh & Lindhard 1920)

At the end of exercise less of the energy is delivered from combustion of carbohydrates than at the beginning as indicated by the decreasing RQ levels and this will require more oxygen

In the present study the cardiac output was maintained constant and the necessary increase in oxygen to the exercising muscle was supplied by an increased extraction of oxygen from the blood i e an increased arterio venous oxygen difference During the type of exercise considered any local demand for an increased flow to comply with the requirement of the heat regulation does not seem to influence the total cardiac output because there is no correlation between changes in cardiac output and body temperature Such local demands may be met by a change of regional perfusion in the intestinal organs such as kidneys and liver Several studies have shown that there is a marked decrease in blood flow to the kidney and to the liver on transition from rest to exercise (Grimby 1965 Castenfors 1967 Wade & Bishop 1962, Rowell et al 1965 Hultman 1966) Some of these authors report values from prolonged exercise which indicate that an additional amount of blood may be shunted from the kidney and liver at the end of prolonged heavy exercise Castenfors (1967) found a decrease of 21 4 per cent in PAH clearance during 45 min supine exercise between period I (0–15 min) and period III (31–45 min) and 16 7 per cent decrease if period III is compared with the mean for periods I and II With a greater thermal load such as exercising in a hot and humid environment the reduction of hepatic flow is greater at the same relative work intensity (Rowell et al 1965)

Heart rate and stroke volume

With a constant cardiac output a rise in heart rate indicates a corresponding decrease in stroke volume The highly significant correlation with a high coefficient of correlation between the increase in heart rate and in body temperature during exercise may indicate a causal relationship between the two variables A study by Christensen (1931) precludes the possibility of such a direct relation because he had subjects who performed prolonged exercise at steady state in respect of heart rate but with an increase of body temperature and, on the other hand subjects exercising at a constant body temperature and increasing heart rate These observations are confirmed by Holmgren s (1956) study of 30 min exercise in well trained men Instead of a direct relation, the correlation indicates the relation both of the heart rate increase and of the increase in body temperature to the relative work load either expressed as pulse rate after 10 min exercise or as per cent of W_{170} The steeper slope of heart rate with time at the beginning of exercise than at the end could be

due to the fact that most of the temperature increase takes place during the first 30—40 min of an exercise period The increase in body temperature during exercise was highly significantly correlated to the relative work load expressed as per cent of W_{170} $r = 0\ 78$ That the total increase in body temperature during exercise is correlated to the relative work load and not to the absolute work load as has been suggested (Nielsen 1938) has been shown recently (Saltin & Hermansen 1966)

Mechanical systole

Previous investigations

The variation in the duration of mechanical ventricular systole has been extensively studied in man by different methods over a long period For references see Strandell (1964 a) who by means of phonocardiography studied the variation of mechanical systole at rest and during stepwise increased exercise in a group of young and old men He found a decrease during exercise which was first linearly related to heart rate, but at high heart rates a significant curvilinearity was recorded with successively lesser decrease of mechanical systole

Present investigation

Mechanical systole measured from the brachial artery pressure curve was obtained from the 12 subjects investigated in the sitting position(I, IV) The values for all individuals were obtained at rest and from almost all subjects during the prolonged exercise every 10th minute From the values at rest and during exercise with the exception of the final values (50 or 60 min exercise) and the corresponding heart frequencies the regression equation for mechanical systole on heart rate was calculated (Fig 3)

Mechanical systole (c sec) $= 37\ 8 - 0\ 119$ heart rate (beats/min) $r = -0\ 93$ (highly significant) $S\ D = 1\ 55$ $n = 63$

This equation is almost identical to that calculated by Strandell for mechanical systole during stepwise increased exercise in young healthy males in the sitting position (1964 a) If the final heart rates in study I are entered in the regression equation and a value for the mechanical systole is calculated the actual mechanical systole measured is longer in 5 of the 6 subjects, mean difference for all 6 subjects 2 03 c sec which is almost significant (see Fig 3) The same relation exists if the calculation is based on the second degree polynomial equation given by Strandell (1964 a) If the final values from study IV also in sitting position but working at lower intensity are compared with the values calculated from the regression equation no significant difference is found In study I the subjects were near exhaustion but in study IV they could have exercised for much longer than one hour

Previous investigations

The variations of the heart volume at rest and during exercise have been studied by many investigators For surveys of the earlier literature see Liljestrand Lysholm & Nylin (1938) and Rushmer & Smith (1959)

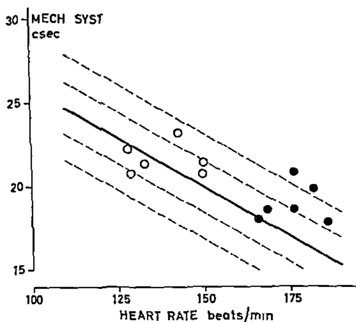


Fig 3 Regression line ± 2 S D for mechanical systole on heart rate at rest and during exercise in the sitting position Open circles are the final values during exercise in study IV Filled circles are the final values during exercise in study I For equation see text

Holmgren & Ovenfors (1960) studied the heart volume in the standing position on transition from rest to exercise in the sitting and supine position and during stepwise increase in work loads both in sitting and supine position They found that the heart volume was 23 2 per cent smaller after 8 min standing compared with supine position On the transition from rest to exercise in the supine position there were no significant changes of heart volume During continued stepwise increased exercise there were no significant changes in the supine position On transition from rest to exercise in the sitting position the heart volume increased by 10 2 per cent The heart volume decreased by 6 5 per cent between the first load with an average heart rate of 99 beats/min and the highest load with an average heart rate of 177 beats/min

Present investigation

In study V the roentgenological heart volume was measured in 10 healthy young men during prolonged exercise of constant intensity in the sitting position and in 6 subjects also in the supine position. The exposure of the roentgen film was made in the end diastole. During the continuous exercise at a constant load there was a continuous decrease of heart volume in both body positions accompanied by a continuous increase in heart rate. There was a significant regression of total heart volume on heart rate with 37.5 ml and 58.3 ml change in heart volume per 10 beats/min change in heart rate in the sitting and the supine positions respectively. The relative work load was 71 and 67 per cent of W_{170} in the sitting and supine positions, respectively. Close to exhaustion the heart volume increased in 6 of 10 subjects in the sitting position by, on an average, 52.5 ml and in 3 of 6 in the supine position by, on an average, 42.0 ml, in spite of a further increase in heart rate.

Comments

Mechanical systole and heart volume. The increase in mechanical systole observed near exhaustion during prolonged exercise in relation to the value expected from the regression of mechanical systole on heart rate during stepwise increased exercise seems to be confirmed by preliminary results from further measurements of mechanical systole by phonocardiography (Ekelund & Odar Cederlof 1967). The difference is probably significant even if one takes into consideration the curvilinear part of the regression line at higher pulse rates. This observation might indicate a change in myocardial function during the actual experimental condition, i.e. at a higher intensity of work for about one hour and continued until exhaustion. The increase in final end diastolic heart volume in some of the subjects (V) is probably the same reaction to the same situation as when the mechanical systole increases.

General discussion

The primary change in the central hemodynamics during prolonged exercise is probably a decrease of the stroke volume, which is compensated for by an increase in the heart rate to keep cardiac output constant. The size of the stroke volume is dependent on the dimensions of the heart and on the emptying and filling of the ventricles. The filling of the ventricles is dependent on the available energy (kinetic and potential) and on the time for filling, and also on the mechanical properties of the myocardium. The filling energy has a static component and a kinetic component. Changes in the distribution of the

blood within the capacity vessels caused, for instance, by gravitation will affect the static component and result in a decrease of the stroke volume. The decrease in stroke volume of about the same magnitude both in sitting and supine position during prolonged exercise of the same relative intensity speaks against gravitation as an important factor in the present material. An estimation of the true pulmonary blood volume during prolonged exercise would have been of interest, but the validity of the methods has not yet been confirmed. The delay in a catheter sampling system when using dye dilution methods introduces a large error which could only be corrected for if the transfer function is known, so the simple subtraction of delay in seconds is unsatisfactory. The calculation of the transfer function and correction could be performed with the aid of a digital computer (Scheel, Langill & Milhorn 1966).

A decrease of the blood volume in the capacity vessels might also be caused by a decrease in the total blood volume. The total blood volume measured with I^{131} albumin also decreases, but the decrease occurs on transition from rest to exercise, and during the prolonged exercise the blood volume is unchanged. That a blood volume decrease of the order of 10 per cent increases the pulse rate during exercise has been shown by Gullbring et al. (1960) after withdrawal of whole blood, and by Danzinger & Cumming (1964) who diminished the plasma volume by 21 per cent in normal subjects with the aid of a diuretic substance. The shift in blood due to vasodilatation caused by the heat regulating mechanism has probably no primary influence on the stroke volume as discussed earlier, under the used experimental conditions.

With the decrease of arterial mean pressure there will also be some chronotropic influence from the baroreceptor system, with an acceleration of the heart rate resulting in a shorter filling period. Influences from the baroreceptor system or from the central nervous system may also have an inotropic effect on the heart muscle, with a change in the myocardial function. For a certain level of work there is probably an optimal combination of filling energy and filling time. A change in one of these components will change the steady state to another level of optimal combination.

The end diastolic dimension of the heart, as measured in study V, decreases when the heart rate increases. During the same period the mechanical systole decreases linearly in relation to the increase in heart rate with the same regression on heart rate as Strandell (1964 a) found during stepwise increased exercise in healthy young men. Those two findings probably indicate that the degree of emptying of the heart is not affected during the first part of the prolonged exercise. In 6 of 10 individuals in sitting position and in 3 of 6 in supine position the end diastolic heart volume increases shortly before finishing the exercise, when the subjects are almost exhausted. In study I the final values

for mechanical systole are probably significantly higher than the values predicted from the corresponding heart rates and the regression equation of mechanical systole on heart rate On the other hand the final values from study IV which was of a lesser degree of intensity fell within the 95 per cent confidence interval of the regression equation of mechanical systole on heart rate These two findings might imply that in some individuals at the end of an exhausting exercise period there may be a change of the mechanical properties of the myocardium accompanied by a less effective emptying of the ventricles

The observed decrease in the systemic arterial mean pressure agrees with earlier observations by Holmgren (1956) and is significant in each study, but is of greatest magnitude during the exercise period at the highest relative work load The decrease would have been of still greater magnitude if the final values had been compared with pressures obtained after 2 minutes exercise because we know from earlier studies (Holmgren 1956 Grimby Nilson & Sanne 1966) that there is also a decrease in arterial mean pressure during the first 10 minutes of exercise In all three studies (I III IV) the decrease in *brachial arterial mean pressure is partly due to a decrease in the systemic* vascular resistance Between 10 minutes and the end of exercise the decrease is not significant in each separate study but if the three materials are lumped together it is significant ($P < 0.01$) The decrease in the brachial artery mean pressure and the systemic vascular resistance is probably due to a decrease in the vasomotor tone Another explanation could be a passive dilatation but the long standing decrease in the brachial artery mean pressure reported by Widimsky Berglund & Malmberg (1963) does not support this assumption

In all three studies (I III IV) there was a decrease in the pulmonary artery mean pressure and also of the calculated pulmonary vascular resistance in the cases when also the pulmonary artery wedge pressure was recorded The total pulmonary vascular resistance consists of a network of resistances in parallel and series functioning in a non linear mode The change of alveolar and intra pulmonary pressure may change the transmural pressure in the pulmonary vessels so that they will pass through the point of critical closing pressure (Permutt & Riley 1963) Such a mechanism will probably operate mostly at the beginning of exercise when the increase in ventilation takes place There was no correlation between change of pulmonary artery mean pressure and of ventilation or respiratory rate

The pressure increase on transition from rest to exercise changes the distribution of blood flow so that the pulmonary vascular bed is more evenly perfused especially in the sitting position (West 1962 Holley et al 1966) Such an opening of the inactive vascular bed may result in a decrease in pulmonary arterial mean pressure on transition from rest to exercise During continuous

exercise however, there is likely to be a more uneven distribution of the perfusion compared with the situation at the beginning of exercise due to an altered distribution of blood within the capacity vessels Such a mechanism would counteract a decrease in pulmonary arterial mean pressure Another factor which may change the pulmonary vascular resistance is a change of the diameters of the perfused vessels, which may be rendered passive by the higher pressure during exercise or active due to a change in the vasomotor tone The passive dilatation of the pulmonary vessels might occur at the beginning of exercise but will probably remain unchanged during prolonged exercise Therefore a change in vasomotor tone seems to be the factor which causes the fall in the pulmonary arterial mean pressure during continuous prolonged exercise The long standing decrease in pulmonary arterial mean pressure after a period of exercise observed by Widimsky et al (1963) supports the latter theory The different distribution of perfusion of the lung vessels in supine and sitting position might be one explanation of the marked difference between the decrease in pulmonary artery mean pressure in the two studies (III IV) which came earlier in supine than in sitting position The difference of sympathetic influence might be another factor which alters the pressure response in the sitting compared with the supine position because the sitting or standing position strongly activates the sympathetic nervous system (Vendsalu 1960)

The influence of exercise on the capacity vessels in the arms has been studied by Bevegard & Shepherd (1965 1966) They studied short time supine exercise and found an immediate constriction of the capacity vessels which persisted during 5 min exercise and was related to the work load Whether such a constriction persists during prolonged exercise is not known from any direct measurements but from the discussion given earlier it seems rather obvious that the tone in the central capacity vessels diminishes during prolonged exercise

CHAPTER IV

Adaptation of the respiration during prolonged exercise

Previous investigations

Christensen & Hansen (1939) studied three normal, healthy males during prolonged exercise for 2—3 hours. The main interest was to study the change in respiratory quotient during exercise. They found during the prolonged exercise an increase in oxygen uptake in all three individuals, mean 11.1 per cent. They published no figures of total ventilation, alveolar ventilation or respiratory rate. In a study by Levy, Tabakin & Hansson (1961) in which cardiac output in normal men during steady state exercise was measured by a dye dilution technique, 12 males worked on a treadmill for 20 minutes. The authors found no change in total ventilation between 5 min and 12 min work. Davies et al. (1965) studied the ventilatory and metabolic response to graduated and prolonged exercise in normal subjects. Three normal subjects worked in sitting position for one hour at 600 kpm/min. There was a slight increase in total ventilation but no figures were given of oxygen uptake. P_{CO} was about 3—4 mm Hg higher during the first 10 minutes of exercise compared with rest and the last period of exercise. P_{CO} was, however, estimated by the Astrup technique and not corrected for the difference between body temperature and the temperature of the pH electrode. In a study by Cobb & Johnson (1963) of prolonged treadmill exercise only an average value for oxygen uptake and ventilation was given.

Present investigation

In all four studies (I—IV) in which oxygen uptake was measured there was an increase during the exercise period; this was most marked in the two studies with the highest relative working intensity (I and II), in which the increase was 7.9 per cent and 11.5 per cent respectively. In the two other studies the increase was of the order of 3—5 per cent. In studies I and II there was a significant increase in ventilation of the order of 18.7 and 19.2 per cent respectively. There was also an increase in alveolar ventilation which, however, was of the same magnitude as the increase in oxygen uptake, 10.0 per cent and 13.4 per cent respectively, so that the increased ventilation must also have been caused

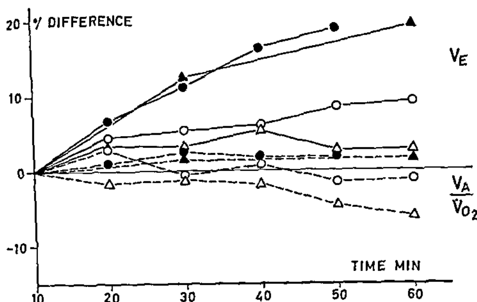


Fig 4 Response of minute ventilation V_E (———) and alveolar ventilation in relation to oxygen uptake V_A/V_O ( ) during prolonged exercise For further data see fig 1 Filled triangles denote values from study II

by an increase in dead space ventilation (Fig 4) In study I the increased dead space ventilation was caused by an increase both in respiratory rate and in dead space volume but in study II it was mainly caused by an increased respiratory rate In the two other studies III and IV there was only a slight but insignificant increase of ventilation The respiratory quotient RQ increased in all studies on change from rest to exercise During the continuous prolonged exercise there was a significant decrease with time The values for RQ during exercise (RQ_e) were highly significantly correlated to the value for lactic acid ($Lact_e$) during exercise the regression equation being

$$RQ_e = 0\,841 + 0\,0259 \quad Lact_e \text{ (meq)} \quad n = 103 \quad r = 0\,64 \quad S\,D = 0\,0323$$

The arterial carbon dioxide tension level was constant during exercise in all four studies (I—IV) The pH of arterial blood decreased slightly in all subjects on change from rest to exercise, then increased slightly during the continuous exercise to about the same value as at rest Corresponding changes were seen in standard bicarbonate with an initial decrease of 0 8—3 0 meq/litre The change of acid base status during the work period seemed to be a result mainly of a change in lactic acid

Lactic acid concentration increased in all cases on change from rest to exercise and then decreased continuously The same pattern was reported by Bang (1936) from lactate studies during prolonged exercise

There was no correlation between the increase in ventilation and in heart rate but there was a significant correlation between increase in ventilation and 10 min heart rate and relative work load expressed as per cent of W_{170} The increase in minute ventilation and dead space ventilation from 10 min to the end of exercise is highly significantly correlated to the corresponding increase in respiratory rate, r = 0 72 and 0 86 The increase in minute ventilation from 10 min to the end of exercise is also highly significantly correlated to the increase in dead space ventilation during the corresponding period r = 0 89 The increase in respiratory rate is not correlated to the increase in body temperature between 10 min and end of exercise This does not confirm the observation of Cotes (1955) who found a relation between body temperature and ventilation during exercise

In study II the steady state diffusing capacity of the lungs for carbon monoxide (DL_{CO}) was measured as well as the other respiratory variables measured in the circulatory studies Between 7 and 30 min work there was a 12 per cent increase in DL_{CO} which was probably significant There was no further change between 30 and 60 minutes The relative level of work intensity was of the same magnitude as in study I about 75 per cent of W_{170} There was a significant increase in ventilation but not of as great magnitude as in study I The increase in total ventilation was effectuated by an increase in rate of breathing while the tidal volume decreased slightly This resulted in an increased dead space ventilation The changes in the physiological dead space were however, less than those observed in study I

Discussion

In the two studies at high working intensity (I and II) there were increases in total ventilation which were due partly to a slight alveolar ventilation corresponding to the slight increase in oxygen main increase was caused by an increased dead space ventilation an increase in respiratory rate In study I there was also an i space volume which might indicate a change in the regional v fusion relationship although the overall ventilation perf (V_A/Q_C) changed only slightly Such a change might ca a decrease in perfusion of the upper parts of the lung exercise which could be expected as a result of the artery pressure There is also a methodological difference

responsible for the difference in dead space ventilation in studies I and II In study I the Astrup technique was used for measuring P_{aCO} with a correction of +3 0 mm Hg to make the values comparable with the electrode values for P_{CO} In the individual case, however the exact value of the correction factor is unknown and small deviations in P_{aCO} cause rather big changes in dead space ventilation (Holmgren 1965a) In studies III and IV there were no significant changes in total ventilation but in the whole material the change in ventilation and respiratory rate was significantly correlated to the relative work intensity but not to the increase in body temperature as has been suggested by Cotes (1955) The increase in total ventilation is mainly caused by an increase in respiratory rate The cause of the variation in respiratory rate is however, not quite clear A stimulation via peripheral chemoreceptors can be excluded because the level of P_{O_2} pH and P_{CO_2} in arterial blood is too stable The change of body temperature does not seem to be of significance in these studies The baroreceptor system especially in the pulmonary vessels, might be responsible for some of the changes in ventilation during the prolonged exercise i e increase of respiratory rate (Kinnison et al 1965) That exercise increases the level of catecholamines in blood has been shown by von Euler & Hellner (1952) by Holmgren (1956) from studies of urinary excretion of catecholamines after exercise and by Vendsalu (1960) from analyses of blood In a metabolic study by Carlsten et al (1965) six young healthy men exercised for 30 min in supine position They found a progressive increase in norepinephrine level in four of the six subjects Intravenously infused norepinephrine increases the ventilation mainly through the increase in oxygen consumption (Havel et al 1964 b Lundholm & Svedmyr 1966) A progressively increased level of catecholamines in blood could therefore be responsible for at least part of the increase in oxygen consumption and ventilation A further explanation is a direct central action as a response to the circulatory changes which Gullbring et al (1960) suggested as being a cause of the increase in ventilation after bleeding A change of the mechanical properties of the lungs as a result of a diminished pulmonary blood volume might be responsible for some of the changes in ventilation during the prolonged exercise The slight change in diffusing capacity during the first part of exercise may be a result of opening of unperfused vessels a mechanism already discussed as responsible for the changes in pulmonary artery mean pressure The initial increase of diffusing capacity and then of the steady level imply that the prolonged exercise does not decrease but rather increases the capillary blood volume

CHAPTER V

General summary and conclusions

The present investigation is concerned with the circulatory and respiratory *adaptation in young healthy men during prolonged exercise of up to one* hour on a bicycle ergometer in the sitting and supine position

Total blood volume

In 18 subjects total blood volume was determined with I^{131} albumin at rest and at the beginning and end of prolonged exercise both in sitting and supine position On transition from rest to exercise there was a decrease of 5 0—9 3 per cent depending on the body position and the relative working intensity During the continuous prolonged exercise the total blood volume and plasma volume were unchanged in all studies

Central circulation

Cardiac output was maintained constant in the different body positions and at different relative working intensities There was a continuous increase in heart rate with a corresponding decrease in stroke volume during the continuous prolonged exercise The decrease in stroke volume was most marked in the study with the highest relative working intensity (I) but there were no differences between prolonged exercise in supine (III) or sitting position (IV) at the same relative intensity There was no significant correlation between the decrease in stroke volume during prolonged exercise and the change in minute ventilation respiratory rate lactic acid concentration after 10 min exercise or decrease in blood volume from rest to 10 min exercise

During the continuous prolonged exercise there was a decrease of the pressures both in the systemic circulation and in the pulmonary circulation The decrease in the systemic circulation was related to the relative working intensity and was caused by a decrease of the peripheral resistance and also by a decrease of the right atrial pressure The decrease of mean pressure in the pulmonary artery was more marked during supine (III) than during sitting exercise (I IV) even at a higher relative work intensity

In the two studies (I, IV) in which the mechanical systole was measured from brachial artery pressure curves both at rest and during the prolonged exercise there was a highly significant regression of mechanical systole on heart rate The final values of mechanical systole were not included in the calculation of the regression equation The final values from the study with the highest working intensity (I) were probably significantly larger than the values calculated from the corresponding heart rates

The end diastolic heart volume measured roentgenologically decreased during the continuous prolonged exercise with a correlation between heart volume and heart rate In 6 of 10 subjects in the sitting position there was a probably significant increase of heart volume immediately before the end of exercise when the subjects were near exhaustion The corresponding figures in supine position were 3 of 6 subjects

The reason for the decrease in stroke volume central and systemic pressures and in end diastolic heart volume is discussed in connection with the deviation of mechanical systole from the calculated values at the end of exercise to exhaustion The findings imply that there is a less effective filling of the heart, due to changes in the available filling energy probably as a result of a decrease in the vasomotor tone At the end of exercise to exhaustion the results imply that there might also be a change in the mechanical properties of the myocardium as indicated by the changes of mechanical systole and end diastolic heart volume at the end of exercise

Respiration

In 3 groups of 6 subjects with different body positions and working intensities there was a continuous increase in oxygen uptake, which caused a corresponding increase in alveolar ventilation Besides there was a further increase in total ventilation as a result of an increased dead space ventilation The increased dead space ventilation was mainly caused by an increase in respiratory rate but in some studies also by an increase in dead space volume which might be a result of a change in the local ventilation perfusion relationship The increased respiratory rate might be a result of reflexes from the baroreceptor system deriving from the receptors in the lung vessels or of an increase of the catecholamine level in the blood The arterial carbon dioxide tension was unchanged during the prolonged exercise The respiratory quotient decreased as did the lactic acid in arterial blood followed by small changes in pH and standard bicarbonate

In the 6 subjects performing sitting prolonged exercise the steady state diffusing capacity for carbon monoxide was measured (II) at the beginning

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ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 294

From the Institute of Physiology University of Lund, Sweden

FACTORS OF IMPORTANCE FOR THE ETIOLOGY OF OBESITY IN MICE

By

STIG LA

LUND

ACTA PHYSIOLOGICA SCANDINAVICA

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FACTORS OF IMPORTANCE FOR THE ETIOLOGY OF OBESITY IN MICE

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RAHMS BOKTRYCKERI AB LUND 1967

CONTENTS

INTRODUCTION

The mechanism of regulation of food intake is complicated and still not completely understood (*cf* Andersson and Larsson 1961 Mayer 1964, and Larsson 1967) Even if many factors are known to influence the feeding mechanisms the nature of the adequate stimulus is not known As a result of the last fifteen years of research in this field, however it has become increasingly obvious that the adequate stimulus for the "feeding center is of multiple origin

It is natural that the nutritional state is dependent on the integrative actions of the feeding mechanisms In this connection the classical experiments on the importance of an intact hypothalamus for the nutritional homeostasis made by Brobeck and coworkers deserve attention (*cf* Brobeck 1955) Thus a prolonged state of hyperphagia caused by bilateral lesions in the ventromedial parts of the hypothalamus leads to over nutrition which in general results in obesity, almost regardless of the composition of the diet at least as long as it fulfills the basic caloric needs (*cf* Brobeck 1955)

Many factors have been found to influence the quantity of the food intake It is generally accepted that under physiological conditions energy intake and expenditure are equal In this connection the quality of the food eaten has to be considered The normal individual keeping constant weight and largely constant body composition will have the ability to adjust the amount of food eaten depending on the quality with regard to protein fat and carbohydrate The accuracy with which nutritional homeostasis is maintained will vary from individual to individual, but will also vary due to the external environment Therefore in order to evaluate the cause of a changed nutritional state hereditary factors must be considered In recent years much interest has been focused on the hereditary obesity in laboratory animals (*cf* Mayer 1964, and Hellman 1965) In animal husbandry the nutritional state of the animals is of fundamental importance and here both hereditary and external factors have to be considered Also in dogs it has been found that hereditary factors are of importance for the etiology of obesity and related diseases (Krook, Larsson and Rooney 1960)

Mayer (1964) differs between regulatory and metabolic obesity, well aware of the fact that such a distinction, although usefull necessitates a certain degree of over simplification It still leaves the question open whether a disturbance in the regulatory mechanism leading to over nutrition will not change the general metabolic pattern so that a metabolic type could be considered

Other factors such as exercise have the ability of influencing food intake (*cf* Mayer 1964, Kennedy 1964 and Parizkova 1966) Also a change of diet in laboratory and domestic animals affects food intake and has a profound influence on the body composition (Ericson, Larsson and Rubarth 1962*b* Clausen 1963, Munck 1964 and Larsson 1966*a*) A laboratory animal when offered a free selection of diet instead of the ordinary laboratory diet might also change in body weight (Larsson 1957) In hereditary obesity in mice it has been found that different diets have profound effects on the development of obesity (Mayer 1964)

The age of the animal has also importance regarding the fat content of the body as well as the fat mobilizing capacity (Benjamin *et al* 1961, Altschuler, Lieberson and Spitzer 1962, and Jelinkova and Hruza 1964)

Thus many factors have been found to exert influence upon the nutritional fate of the organism As has been pointed out, the feeding mechanisms *per se* cannot be studied and discussed separately without taking into account the contribution of many factors which acting together, maintain a nutritional homeostasis in the normal individual (Larsson 1967)

The present study was made with the purpose of comparing the hereditary factors in various strains of mice in relation to external factors such as exercise and different types of diets for the etiology of obesity

CHAPTER I

General material and methods

In the present study mice from three different strains were used *viz* the Swiss NMRI and CBA strains, which were originally obtained from the Institute of Genetics (L Munck) University of Lund, and then bred at the Institute of Physiology.

Except when otherwise stated, the animals were fed *ad libitum* and given a pellet diet. The composition of the food may be seen in Table I A and B. The mixing and pelleting of the food was done by AB Teknosan Malmo. The food was bacteriologically controlled and stored at +4°C.

The mice had free access to tap water. The animals used for breeding received in addition to the standard diet an extra supply of calcium phosphorus and vitamins. The litters were weaned when 22 days old.

The animals were generally kept in macrolon cages with wood shavings. When the mice were starved not only the food but also the wood shavings were taken away.

The size of the cages varied according to the experimental set up and will be given for each experiment. If not otherwise stated the animals were weighed once a week.

Experimental obesity in the various strains of mice was produced by a single intraperitoneal injection of goldthioglucose (Solganol B oleosum Schering). To obtain maximal efficiency of the injections with regard to the development of obesity the Swiss mice were usually injected in the fed state with 0.8 mg goldthioglucose per g body weight. The NMRI and CBA mice were injected after 24 h fast with 1.5 mg and 0.5 mg per g body weight respectively. The reason for the different doses used will be discussed in Chapter V.

When blood samples were taken the technique described by Rerup and Lundquist (1966) was applied. This method is based on orbital bleeding by means of constricting pipettes and allows daily blood sampling of at least 25 µl without upsetting the basic physiological conditions. It is furthermore a very rapid way of blood sampling.

TABLE I A

The composition of the standard food used in the experiments

Content per kg food	g			mg
Barley	400	Vit B		20
Oats	120	Vit B_6		5
Wheat	100	Ni otylamide		40
Lucerne	30	Folic acid		0 2
Meat meal	50	Calcium panthothenate		60
Fish meal	60	Choline bitartrate		40
Dried skim milk	34	Vit A	30 000 IU	
Dried pig blood plasma	10	Vit D	3 000 IU	
Casein	10	Vit E		70
L lysine HCl	1 5	*Ferrosulphate*		500
DL methionine	0 5	Manganese sulphate		200
Lard	6	Copper sulphate		175
Glucose	10	Cobolt sulphate		60
Dried brewer s yeast[1])	25	Zink sulphate		50
		Magnesium sulphate		100
		Sodium chloride		5000

[1]) Composition of the dried brewer s yeast Content in % Protein 50 carbohydrates 35 ash 8 and water 7

Vitamin content (μg/g) B_1 120—150 B 30—50 Nicotinic acid 400 Pyrodoxine 50 Folic acid 30 B_1 not known Pantothenic acid 200 Choline 3500 Inositol 3000 Biotin 1 and Para aminobenzoic acid 10

TABLE I B

General characteristics of the standard food used in the experiments (in %)

Raw protein	23 7	Digestible protein (determined with pepsin and HCl)	18 6
Raw fat	4 0	Non digestible protein	1 5
Nitrogen free extractable substances	50 6	Amides	3 6
Crude fibre	4 5	Calcium	1 36
Ash	7 5	Phosphorus	1 08
Water	9 7		
	100 0		

Incubation of adipose tissue in vitro

In order to study the net release of glycerol and free fatty acids (FFA) from adipose tissue *in vitro* the following general procedure was followed

The mice were killed by cervical dislocation About 50 mg from each side of the epididymal fat pad was carefully removed

The fat was incubated in a Dubnoff type metabolic shaker at 37°C in Hagerdorn tubes for one hour with and without adrenaline (1 μg/ml) in 4 ml Krebs Ringer phosphate buffer to which was added 2 % albumin (bovine serum albumin Sigma) The incubation was carried out in air The pH of the incubation fluid at room temperature was 7 40—7 43 The ionic concentration of the buffer was the same as that used by Munkner (1963), containing NaCl 120 mMol/l KCl 5 6 mMol/l CaCl 2 6 mMol/l KH_2PO_4 1 2 mMol/l, $MgSO_4$ 1 2 mMol/l and Na_2HPO_4 10 mMol/l

Particularly in studies concerned with the inhibition of lipid mobilization *in vitro* bicarbonate buffer is as a rule preferred to phosphate medium Thus Martin, Horning and Vagelos (1961) have found that bicarbonate ions are essential for normal lipid synthesis

When bicarbonate buffer was used and the results of the release of glycerol and FFA compared with phosphate medium no differences were observed

A small portion of the gonadal fat was taken out from the mice and immediately frozen for further analysis on the content of glycerol and FFA

After incubation the adipose tissue was cleaned in ice cold isotonic sodium chloride and frozen The incubation fluid was also frozen

In each experiment the albumin containing medium was analyzed for FFA As a rule no glucose was added to the medium

Addition of glucose to the medium has been found to reduce the release of FFA (Lopez, White and Engel 1959 and Raben and Hollenberg 1960) Since the total FFA content of the system decreases, one must assume an enhanced esterification Further, glucose in the medium will not cause any change in the release of free glycerol (Vaughan 1962)

Determination of FFA in blood plasma, incubation medium and adipose tissue

The method used was essentially the same as that published by Laurell and Tibbling (1967) Thus 0 1 ml plasma 0 5 ml medium or 0 2 ml homogenized adipose tissue (1 20) were shaken with 1 ml 1/15 M phosphate buffer pM 6 8 and 6 ml chloroform — heptane (4 3 v/v) After centrifugation the upper phase was discarded The chloroform heptane phase was shaken with an alkaline coppernitrate ethanolamine solution saturated with NaCl in order to obtain the chloroform heptane mixture in the upper

phase The FFA in this phase will participate in a FFA copper complex in proportion to the FFA concentration Part of the upper phase is used together with diphenylcarbazide which forms a redish copper complex, the extinction of which is measured at 540mμ Palmitic acid is used as the standard This method gives good reproducibility and seems to be more sensitive and specific than other methods described in the literature

Thus samples analyzed after this method showed close correlation with samples analyzed when run on silica gel)

The test tubes used in the extraction procedure of the FFA and the pipettes used were silicon treated which seemed necessary in order to get reproducable values

Determination of glycerol in blood plasma incubation medium and adipose tissue

Glycerol was determined according to Laurell and Tibbling (1966) using an enzymatic fluorometric method allowing measurements of very small amounts

The method is a fluorometric adaptation of the enzymatic conversion of glycerol where reduced diphosphopyridine nucleotide is formed in proportion to the glycerol concentration The samples were read in a spectro-fluorometer at exciting wave-length at 350 mμ and emitted at 458 mμ An Aminco Bowman instrument was used for these measurements

Blood sugar was determined by the method by Marks (1959) with 0 33 M perchloric acid buffered with glycine to give pH 2 7, as the protein precipitant

Determination of carcass content of water protein and fat

After the adipose tissue samples had been taken, the gastro intestinal content was removed by washing and the animals frozen The carcass content of water protein and fat was determined as described previously (Larsson 1966*a*) Thus the mice were homogenized *in toto* with water in a MSE homogenizer An aliquot of the homogenate was further homo-

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*) These determinations were made by Dr Laurell and Dr Tibbling Department of Clinical Chemistry University of Lund

genized and diluted with water for protein analysis according to Lowry et al (1951) The total mouse homogenate was dried to obtain the water content of the body employing the calculation formula used by Munck (1964)

$$X = \frac{(I-U)\ (M+V)-I\ V}{I\ M}\ 100$$

X = water content of carcass in %
V = added water (ml)
M = weight of mouse (g)
I = weight of homogenized animal (g)
U = weight of dry substance (g)

The dried homogenate was then ground and extracted with petroleum-ether in a Soxleth apparatus for 10 hours to obtain the fat content

Biological definition of the standard food

As may be seen from Table I A and B the protein content of the standard laboratory food generally used in the present study is comparatively high As shown by Ericson Larsson and Lid (1961), however it is not the protein quantity that determins the growth rate and food utilization of a diet Rather it is the protein quality that is largely responsible for the high values of these parameters By measurements of the protein efficiency ratio (PER*) and by carcass analysis of the content of fat and protein one can largely judge the quality of the food

For this purpose newly weaned (22 days old) male mice of the CBA strain were given the standard food for three weeks The weight of the

TABLE II

Growth, protein fat ratio and PER in male CBA mice on the standard laboratory food and on a reference cereal diet (50 animals in each group)

	Growth in g	Protein — fat ratio	PER
Standard lab food	14.5	2 12	2.70
Cereal food	8.7	0 96	1 40

*) PER = growth per g protein consumed.

animals was measured once a week as was the food intake For comparison, a diet containing only the cereal components supplemented with the vitamins and minerals shown in Table I A was fed to another group

After three weeks on the diets the animals were killed by cervical dislocation and the carcass content of protein fat and water was determined as described previously in this chapter

Table II shows the results of this introductory study and reveals that the standard diet gives high growth rate and protein fat ratio

Expression of data

All experimental evidence indicates that fat is mobilized from adipose tissue probably entirely in form of FFA During incubation *in vitro* the fatty acids are released into the medium However, the fact that the tissue content of FFA may undergo changes suggests that the tissue content of FFA before and after incubation must be of importance to access the net release of FFA Thus Vaughan (1962) has found that FFA may appear in the medium as a result of transfer from the tissue FFA compartment not necessarily parelleling the net generation of FFA in the system Of importance in this connection is that all albumin preparations contain a significant amount of FFA, unless specially pretreated (Goodman 1957) This requires, of course that the zero time FFA content will be measured both in the incubation medium and in the adipose tissue

It is obvious that due to the fact that FFA production can be influenced by changes in the rate of triglyceride synthesis (resynthesis of released FFA) as well as by changes in the rate of lipolysis true information of lipid mobilization cannot be obtained by using FFA net release as the only parameter (*cf* Steinberg and Vaughan 1965)

As studies have shown that free glycerol is metabolized only very slowly by adipose tissue (Shapiro Chowers and Rose 1957 and Steinberg and Vaughan 1961) due to absence of demonstrable phophoglycerokinase activity (Wieland and Suyter 1957, and Margolis and Vaughan 1962) measurement of net release of glycerol is considered to be a safer parameter for the fat metabolism *in vitro* (*cf* Steinberg and Vaughan 1965) However changes in the fate of glycerol in adipose tissue must be kept in mind although they are regarded as less important when compared with changes in net release of FFA (*cf* Steinberg and Vaughan 1965)

The average reading when 18 samples with 10 μM palmitic acid were determined was $0\ 623 \pm 0\ 0044$ whereas the corresponding figure when the same number was determined on 18 consecutive days was $\pm 0\ 0073$

The average reading when 20 samples with 0.4 mM glycerol were determined was 0.220 ± 0.0027, and when the same number determined on consecutive days was ± 0.0050.

The problem as to the correct way of expressing the data from the metabolic events of the adipose tissue can be discussed indefinitely. Thus Hellman, Larsson and Westman (1962) pointed out the importance of expressing metabolic data in relation to the number of cells, i.e. metabolic units, and also emphasized the importance of considering the biochemical data with regard to morphological findings. This statement became more evident when it was shown that the number of mast cells greatly increased in adipose tissue in obesity in mice (Hellman, Larsson and Westman 1963). Therefore it was felt wrong to express data in terms of nitrogen or DNA in the present study. Also the expression of data in terms of number of fat cells could be irrelevant, since also the mast cells could be regarded as having a metabolic activity of their own. Thus the data in the present study regarding the *in vitro* experiments are expressed as units per g weight, although it might be regarded as a certain degree of over simplification.

Criteria and definition of obesity

In the literature there is no general agreement as to the definition of obesity. Therefore, the dividing line between leanness and obesity will be arbitrary. Fenton and Dowling (1953) have suggested a criterion of obesity based upon the relative amounts of body fat and of the fat free weight compartment. Thus, by plotting carcass fat against fat free weight for individual animals at a certain age one obtains a certain regression line, the slope of which eliminates the obese individuals. Such a way of defining obesity — as a marked deviation from the regression line — is no doubt very suitable when the mice are not too old. Many older mice, as will be shown later in this study, however, will develop "age obesity".

Figure 1 shows the relation between fat and fat free body weight in the Swiss NMRI and CBA mice. The broken lines represent the regression line plus minus twice the standard error. Above this the animals are regarded as obese.

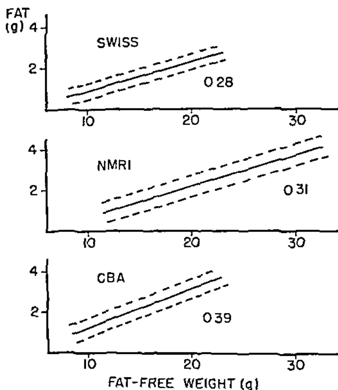


Fig 1 Regression line for fat against fat free weight in Swiss NMRI and CBA mice 22—180 days old The mice have been given the standard laboratory food The slope of the line is given in the figure Broken lines *indicate twice the standard error of* estimate In each strain about 200 animals have been used

CHAPTER II

"Lack of success in the treatment of obesity requires that we maintain a flexible attitude towards these patients
Gordon 1954

SOME CHARACTERISTICS OF SWISS, NMRI AND CBA MICE

by

STIG LARSSON and SVEN JONSSON

In line with the preliminary study demonstrating strain differences in mice with regard to decrease in weight, change in body composition and in FFA of blood plasma after 24 hours of fasting (Larsson 1966 *b*), it seemed appropriate to obtain more information on these differences

As briefly reviewed in the introduction, many factors are thought to contribute to nutritional homeostasis The influence of these could, however be expected to have different importance in various species and strains within a species

Lyon and Fenton (1956) have reported that the levels of glycogen in liver and muscle differ in various strains of mice in relation to inherited differencies in metabolic and endocrine patterns The feeding of diets with high fat contents can induce obesity in some strains of mice but not in all (*cf* Fenton 1960) Such breed characteristics have also been observed in dogs with regard to the occurence of obesity and diabetes mellitus (Krook *et al* 1960)

In the obese state it has been shown that the FFA content of adipose tissue will vary in rise after starvation in different types of obesity in mice (Hollifield Perlman and Parson 1962) It could therefore, be possible that in the different strains of mice the glycerol and FFA content in and release from adipose tissue could be different

The present experiments were made to further examine the observed strain differences with regard to different parameters beleived to be of importance for nutritional homeostasis and thus for the etiology of obesity

Material and Methods

Mice of the Swisse NMRI and CBA strains were used They were as usual fed *ad libitum* with free access to tap water The food was the standard diet described in Chapter I

At the end of the experimental period the animals were killed and analyzed with regard to the carcass content of water protein and fat as described in Chapter I

Experiment 1 From each strain 10 mice were starved for 24 hours at eleven different ages The weight loss was measured and the animals killed

by cervical dislocation Part of the epididymal fat was incubated as described in Chapter I The same procedure was made in mice in the fed state Groups of mice from each strain were also starved for 6 hours and treated as above

Experiment 2 Goldthioglucose obesity was produced in the Swiss, NMRI and CBA strains as described in Chapter I The criteria for obesity were the same as outlined in the same chapter The animals were not used in the experiment proper until they had reached the static phase of obesity namely, they did not increase appreciably further in weight and/or fat content on the standard laboratory food, after having passed the dynamic phase of obesity At this time they were on the average 95 days old Groups of the obese mice were starved for 24 hours and the decrease in weight measured After killing the animals by cervical dislocation, they were treated as described in Chapter I regarding carcass analysis and the net release *in vitro* of glycerol and FFA from adipose tissue

Experiment 3 Mice of the Swiss NMRI and CBA strains at the ages of 33 95 and 180 days were used After 24 h of food deprivation in half of the animals they were subjected to blood sampling as described in Chapter I and the blood analyzed with regard to the content of blood sugar as described in the same chapter

Results

Experiment 1 Figure 2 shows the decrease in weight of mice of different strains at various ages after 24 h deprivation of food It may be noted that young Swiss mice decrease up to 30 % of the body weight during this time while the NMRI and in particular the CBA mice exhibit a smaller decrease in weight at the same age In common for all three strains the decrease in weight is less pronounced with increasing age (Figure 2)

The decrease in weight after 24 h of fasting is followed by a marked decrease in body fat content which may be seen in Figure 3, where the fat content is plotted against the fat free weight (see Chapter I) The decrease in body fat largely follows the same characteristics in strain and age as mentioned for the weight decrease However new regression lines for the slope after 24 h fast in the various strains can be drawn (*cf* Fig 1)

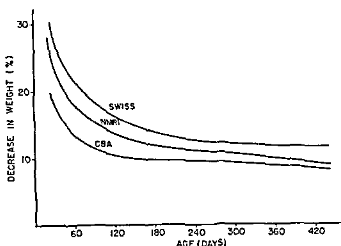


Fig 2 Decrease in weight (%) after 24 h fast in male Swiss NMRI and CBA mice at different ages

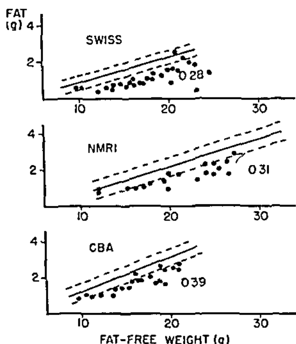


Fig 3 Carcass fat plotted against fat free weight in Swiss NMRI and CBA mice after 24 h fast (solid circles) in relation to corresponding values in the fed state as indicated in Fig 1

Table III shows the content in epididymal fat of glycerol and FFA of mice in the fed state and in those starved for 6 h and 24 h Figures 4 and 5 others have been expressed in the same way as used by Munkner (1963) for other parameters As may be seen from the Table and Figures 4 and 5 certain differences exist Thus, there is a difference in the glycerol content of the adipose tissue in Swiss mice between the values from 24 h fast and those obtained in the fed state and after 6 h fast The same tendency is met regarding the NMRI and CBA mice (Figure 4) Among the different strains there is only a difference after 24 h fast between Swiss mice and NMRI and CBA mice As may be seen in Figure 5, the FFA

TABLE III

The content of glycerol and FFA in the epididymal fat pad of male Swiss NMRI and CBA mice in the fed state, and after 6 and 24 h of fast The age of the animals is between 90 and 140 days Mean±s e m ( )=number of animals Differences in Fig 4 and 5

Strain	Glycerol μM/g adipose tissue			FFA μM/g adipose tissue		
	Fed	6 h fast	24 h fast	Fed	6 h fast	24 h fast
Swiss	3 3±0 40 (12)	2 9±0 37 (7)	8 0±0 48 (7)	0 7±0 04 (13)	1 3±0 10 (9)	2 1±0 14 (8)
NMRI	4 1±0 44 (11)	3 6±0 24 (8)	5 5±0 29 (10)	0 4±0 04 (10)	1 0±0 04 (8)	1 7±0 07 (8)
CBA	3 6±0 57 (11)	3 3±0 29 (8)	5 7±0 34 (5)	0 3±0 05 (14)	0 7±0 04 (10)	1 5±0 06 (8)

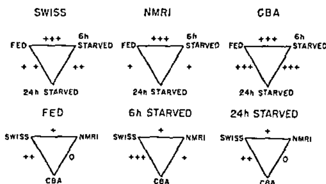


Fig 4 Differences in glycerol content of epididymal fat pad in male Swiss NMRI and CBA mice fed and after 6 or 24 h fast calculated according to Student s t test, after the values in Table III +++=p<0 001 ++=p<0 01 +=p<0 05 and 0=no difference

content in adipose tissue differs significantly in each strain due to the length of food deprivation Among the three strains there are significant differences except between NMRI and CBA mice in the fed state and after 24 h fast

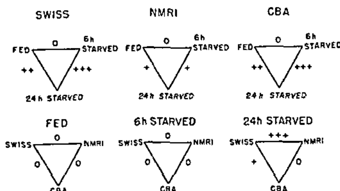


Fig 5 Differences in FFA content of epididymal fat pad in male Swiss NMRI and CBA mice fed and after 6 or 24 h fast calculated according to Student s t test after the values in Table III $+++=p<0.001$ $++=p<0.01$ $+=p<0.05$ and 0=no difference

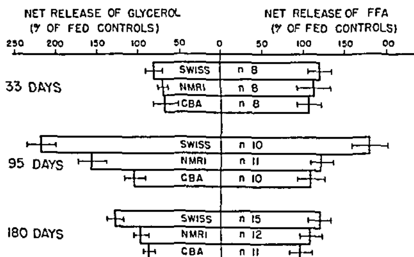


Fig 6 The net release of glycerol and FFA from epididymal adipose tissue in male Swiss NMRI and CBA mice fasted for 24 h compared with fed animals at three different ages The differences are calculated ±s e m in % deviation from the corresponding fed mice

Figure 6 shows the net release of glycerol and FFA from adipose tissue *in vitro* in mice starved for 24 h from three age groups, compared in percent with the values of fed litter mates From this figure it may be noted that in the older groups of mice the glycerol values differ significantly while in the 33 days old mice there is no significant difference Among the various age groups, it may also be seen, that it is only the Swiss mice which have a significantly higher FFA net release compared with the two other strains

A response to adrenalin was noted but followed the same magnitude, relatively as without this substance

Experiment 2 It may be seen from Table IV that starvation of obese Swiss and NMRI for 24 h causes significantly less weight loss than in corresponding lean animals The 24 h fast increased the glycerol and FFA content in adipose tissue significantly in obese Swiss mice compared with the corresponding fed (obese) animals (Table V)

TABLE IV

The effect of 24 h of fast in male obese Swiss NMRI and CBA mice with regard to decrease in weight (in % to non fasted animals) compared with corresponding values in fasted non obese mice Mean±s e m Differences calculated according to Student s t test a)=p<0 001 b)=p<0 01

	Swiss	NMRI	CBA
Controls	18±06 [a]	15±0 4 [b]	11±0 3
Obese	14±0 4	12±0 5	10±0 8

TABLE V

The content of glycerol and FFA in the epididymal fat pad of obese fasted and fed Swiss NMRI and CBA mice Mean±s e m ( )=number of animals Differences calculated according to Student s t test a)=p<0 001 b)=p<0 01 and c)=p<0 05

Strain	Glycerol μM/g adipose tissue		FFA μM/g adipose tissue	
	Fed	24 h starved	Fed	24 h starved
Swiss	1 4±0 05 [b] (13)	2 0±0 19 (8)	0 4±0 05 [a] (13)	0 7±0 05 (8)
NMRI	1 5±0 11 [a] (9)	2 1±0 18 (9)	0 3±0 03 (9)	0 4±0 04 (9)
CBA	1 3±0 10 (10)	1 6±0 12 (9)	0 3±0 04 (10)	0 4±0 04 (9)

The net release of glycerol and FFA from the adipose tissue *in vitro* due to the starvation for 24 h did not increase significantly in either of the three strains even if there was a tendency towards higher values. The response to adrenalin *in vitro* followed exactly the same pattern.

Experiment 3. When normal mice were starved for 24 hours there was a significant decrease in the blood sugar values. The decrease was particularly obvious in the Swiss mice at any age, even if changes also took place in the two other strains after starvation (Table VI).

TABLE VI

Plasma glucose of normal male Swiss, NMRI and CBA mice at three different ages in the fed state and after 24 h of fast. Mean±s.e.m. 45 animals in each group. Differences calculated between fed and starved animals according to Student's t test. a) = $p < 0.001$

Strain		Plasma glucose mg/100 ml of blood		
	Age (days)	33	95	180
Swiss	Fed	123±4.8 a)	129±6.0 a)	128±5.5 a)
	Starved	47±2.5	61±3.8	72±3.6
NMRI	Fed	121±6.9 a)	124±7.2 a)	126±6.8 a)
	Starved	82±5.8	91±4.9	90±5.4
CBA	Fed	127±4.9 a)	129±6.4 a)	125±4.8 a)
	Starved	85±5.7	89±7.7	94±6.2

Discussion

The results obtained from experiment 1 in this chapter clearly indicate strain differences. Without food for 24 hours the young Swiss mice had the heighest weight losses in %. In these animals the 24 hours of fasting also have a profound effect on the blood sugar level which goes down to almost hypoglycaemic levels (Table VI). This finding confirms the previous preliminary experiments (Larsson 1966*b*). The fat content of the carcasses of the young Swiss mice was extremely low after the 24 h deprivation of food. In the youngest mice it was even impossible to get enough fat for incubation. In a study of the effect of body weight on free fatty acid release by adipose tissue *in vitro* it was found that the youngest, most rapidly growing rats released the most FFA per unit weight of tissue (Altschuler *et al.* 1962). Further, with increasing age the responsiveness of

adipose tissue to adrenalin *in vivo* and *in vitro* decreases as measured by the release of FFA (Benjamin *et al* 1961, Dury 1962 and Jelinkova and Hruza 1964) These findings could not be supported by the present study On the other hand, the mice studied in the present investigation were not as old as the rats used in the previous studies Further, it has been shown that probably the measurements of glycerol is more adequate *in vitro* as *in vivo* (Chapter I)

It can be discussed whether the adipose tissue of obese mice with respect to the metabolism of glycerol and FFA does not behave like adipose tissue in old mice In this respect it is necessary to point upon the results from starvation Thus, Chlouverakis (1962) found that the adipose tissue content of lipoprotein lipase significantly dropped in rats with increasing age In the present study very old mice have not been studied On the other hand the present results indirectly confirms this observation Modern research with a tendency to use radioactive compounds to study metabolism of this sort has a tendency to overlook the possibility to use parameters such as body weight and fat content of the body By using these measurements one will assure more accurate determinations of the total effect of a "physiological situation when studying nutritional homeostasis

The differences due to the strains considered seem to be of importance Cheek and Holt (1963) studied the growth and body composition of male albino mice They found that the weight of the mice when plotted against the age gave an S shaped curve regarding growth The same curve was found when fat and water contents were plotted against age This way of expressing obesity might be very useful but as has been shown it is not the right evaluation of obesity (Larsson 1966*a*) In certain types of adiposity overweight is not found but a relative increase of body fat with age Such data has been given for animals (Moulton 1923) and for man (Brozek 1952) Other studies in man seem to point in the same direction (Keys 1964) The present results are largely in agreement with the previous findings Between the different strains however marked differences were noted with regard, in first hand to the reaction towards 24 h of fast

Marshall (1961) studying the influence of adrenaline and fasting on FFA mobilization in goldthioglucose induced obesity found that FFA production was similar in adipose tissue of fasted obese and lean mice This observation is only partly in agreement with the results of the present study Rather the results in this investigation are more like those obtained by Hollifield *et al* (1962) They studied the FFA content of adipose tissue

in three types of obese mice during fasting and found that in goldthio glucose obese mice, the adipose tissue content of FFA was lower than in corresponding controls

When the carcass fat content of the mice starved for 24 h is plotted against the fat free weight one gets the impression that a new regression line can be drawn from that in fed animals as described in Chapter I The slope will be lower in the Swiss mice than in the two other strains, indicating a more active fat mobilization As mentioned in the beginning of this discussion this chapter indicates definite strain characteristics regarding the mice of the different strains It is therefore logical that many non confirming observations from laboratory to laboratory which are not in the same line could depend on this strain phenomenon The present results show that hereditary factors play an important role for the nutritional fate of the mice in the present study with regard to the deposition of body fat and thus being a factor of importance for the etiology of obesity

CHAPTER III

"No pig can form lean meat up to the limit determined by its hereditary unless its diet has sufficient quantities of protein of high biological value and no pig can be forced, by means of extraordinary high levels of protein in its diet to produce more lean meat than permitted by its hereditary

Clausen 1963

INFLUENCE OF PROTEIN CONTENT AND QUALITY IN THE FOOD UPON GROWTH, BODY FAT CONTENT AND FOOD EFFICIENCY IN VARIOUS STRAINS OF MICE

by

STIG LARSSON

In recent years several studies have demonstrated the importance of the protein quality for growth and feed efficiency in animals By these studies it has become evident that it is not the protein content of the food *per se* that determines the biological value, but rather the optimal participation of essential amino acids (Ericson *et al* 1961 and Munck 1964) The processing of natural proteins often decreases the quality as measured by the growth rate and food utilization due to a decrease in the availability of certain essential amino acids (*cf* Ericson *et al* 1961 Ericson and Larsson 1962, and Larsson 1966 *a*) This is of importance in animal husbandry and many studies have been made to determine the effect of amino acid supplementation to foods used, for example in pig breeding (*cf* Clausen 1963, Tollersrud 1961, and Larsson Nilsson and Olsson 1966)

A low biological value of the food protein has a tendency to increase the fat content of the body (Clausen 1963 Munck 1964, Larsson *et al* 1966 and Larsson 1966*a*) It has therefore been suggested that the protein quality of the food is of certain importance for the etiology of obesity (Larsson *et al* 1966 and Larsson 1966*a*)

Of natural reasons it ought to be pointed out that supplementation of low quality protein with different protein "concentrates has been practiced for many decades

The present study was made to get more information as to the relation between the quantity and quality of the food protein and the development of nutritional obesity in different strains of mice

Material and Methods

Twenty two days old male mice of the Swiss, NMRI and CBA strains were used in the experiments The various diets were given the animals *ad libitum* with free access to tap water The mice were kept in macrolon cages 10×16 cm in size

When the feeding period was ended all mice were killed by cervical dislocation The carcass content of water protein and fat was then determined as described in Chapter I

TABLE VII

The composition of the three diets used in Chapter III Experiment I.

	Cereal food (A)	Group I (B)	Group II (C)
Dried skim milk			50 0
Fish meal		40 0	25 0
Sucrose	10 0	10 0	10 0
Dried brewer s yeast	5 0	5 0	5 0
L lysine HCl (feed grade quality)		3 8	1 5
DL methionine		1 3	0 5
Wheat meal	3 3	35 3	3 3

In all three groups the final food had the following composition

Concentrate (as listed above)	10
Oatmeal	15
Barley	30
Wheat meal	24
Wheat bran	21
	100 kg

Experiment I The composition of the three diets used in this experiment may be seen in Table VII The vitamin and mineral supplementation of the diets was the same as in the standard laboratory food (Chapter I, Table I A) The content of digestible protein (in %) as judged by measurements after treatment with pepsin HCl was for the cereal diet 9 3 group I 11 6 and group II 12 4 The fat content of the three diets was (in %) 4 1 4 2 and 3 9 respectively The feeding period lasted for three weeks

The weight of the mice was measured once a week

TABLE VIII

The composition of the three diets used in Chapter III Experiment II

	Protein	Starch	Corn oil	Cellulose
Casein	100	550	50	300
Egg albumin	100	550	50	300
Wheat protein	100	550	50	300

Experiment II Also in this experiment three different diets were given the mice The composition of the diets is given in Table VIII The protein contents (in %) of the three diets were for "casein 11 0 "egg albumin 10 1 and for "cereals 9 4

Results

Table IX indicates protein — fat ratio of the carcass after feeding the different diets for three weeks It may be noted that the effect of the diets varies depending upon the strain of mice considered Among the diets there is a considerable difference between diet A *vs* diets B and C In general, diet C gives the highest protein — fat ratio, regardless of the strain

TABLE IX

The protein fat ratio of the carcass in Swiss NMRI and CBA mice after feeding the three diets the composition of which is given in Table VII

Strain	Diet A	Diet B	Diet C
Swiss	1 9	2 4	2 6
NMRI	1 8	2 3	2 5
CBA	1 0	1 8	2 1

The results from experiment II may be seen in Table X The PER values indicate that in all strains there is a difference between the "wheat diet and the two other diets (Fig 7) The body fat determinations also found in table X indicate that in the Swiss mice no difference exist between the groups on the various diets Figure 7 indicates however that the body fat contents of the NMRI and CBA mice vary due to the diet given

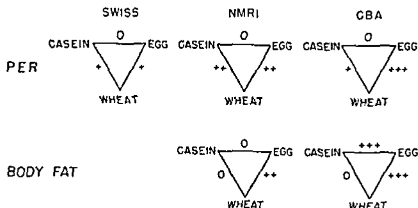


Fig 7 Differences in PER (protein efficiency ratio) and in body fat content of Swiss NMRI and CBA mice *given three isocaloric diets only varying in type of protein* Differences calculated after the values obtained in Table X according to Student's t test +++ = $p<0.001$ ++ = $p<0.01$ + = $p<0.05$ and 0 no difference

TABLE X

The effect of the diets given in Table VIII on growth in g per g protein (PER) body fat content and protein fat ratio in Swiss NMRI and CBA mice Mean ± s e m 40 animals in each group Differences in Fig 7

Diet	Growth in g per g protein consumed			Body fat content %			Protein — fat ratio		
	Swiss	NMRI	CBA	Swiss	NMRI	CBA	Swiss	NMRI	CBA
Casein	2 8±0 15	2 9±0 18	2 1±0 16	10 1±0 82	11 0±0 83	13 0±0 69	2 0	1 6	1 0
Egg albumin	2 8±0 14	3 1±0 19	2 5±0 15	10 0±0 77	9 5±0 80	9 5±0 72	2 2	2 3	2 1
Wheat protein	2 3±0 18	2 0±0 71	1 6±0 17	11 5±0 88	12 5±0 72	14 0±0 83	1 7	1 3	1 1

Discussion

The results from this Chapter indicate that apart for the strain differences which again can be noted, the type of protein used in the various diets is of fundamental importance for the fat deposition in the body

It has been shown in several studies that when cereal products are used as basal food lysine is generally the first limiting amino acid with regard to the biological value of the protein when the interest is focused towards growth and protein efficiency (Ericson *et al* 1961, Ericson Larsson and Östholm 1962*a*, Clausen 1963 and Larsson 1966*a*) Also when consideration is taken into the effect of body fat deposition is is found that increased biological value of the protein in the food will decrease the body fat content (Ericson *et al* 1962*b* Clausen 1963, Munck 1964, Larsson 1966*a* and Larsson *et al* 1966) All these experiments were performed under strict conditions with regard to the food given The basal food also was of vegetable origin Usually the laboratory diets are based on cereals which are supplemented with "high quality proteins, usually of animal origin This is also the case for foods used in animal husbandry During the preparation of food in particular lysine can be lost by different types of reactions Thus combination with other amino acids may give rise to unnatural peptide bonds involving the ε amino group of lysine from which the lysine can be released by acid hydrolysis but not by digestive enzymes The ε amino group of lysine can also be combined with aldehyde groups of sugars or other compounds from which it in some cases can be recovered after acid hydrolysis

In experiment I in this chapter two of the diets were supplemented with animal proteins The protein content of these diets could be regarded as comparatively low but of high quality On basis of earlier findings and analysis with regard to the content of lysine and methionine these amino acids were added (*cf* Ericson and Larsson 1962 and Larsson 1966*a*) The mice were only given the diets for three weeks which is enough to cause changes in the protein — fat ratio The Swiss and NMRI mice seem to react similarly to the diets while the CBA mice tend to be more sensitive to the improvement of diet A by the increased quantity and quality of the protein resulting in diets B and C In this experiment food consumption and utilization was not measured since the primary interest has been to study changes in the protein — fat ratio due to the strain and diet used In experiment II the three diets were isocaloric with protein contents as equal as possible Any change in the results thus was dependent on the quality of the proteins, within the particular

strain The feeding period was only two weeks, because it has been shown that the PER values of animal protein will decrease faster with time compared with cereal protein (Morrison and Campbell 1960) Further introductory experiments gave evidence for a faster decrease in PER in the NMRI mice than in the other strains Table X shows that the PER values between the casein and eggalbumin diets do not differ (Fig 7) On the other hand the wheat diet has lower PER than the two other foods It is evident that the explanation for this difference is due to a lack of lysine and partly of threonine since these to amino acids have been shown to limit the biological value of wheat (Ericson *et al* 1961 and Larsson 1966*a*) The feeding period was too short to expect the development of nutritional obesity to any extent On the other hand, if the body fat is plotted against the fat free body weight as described in Chapter I (Fig 1) one will find that the NMRI and in particular the CBA animals on the wheat diet will fall above the regression line In this connection it is of interest to note that the two experiments in this chapter seem to indicate that the CBA mice are very sensitive to the protein quality with regard to the parameters studied while the Swiss mice on the wheat diet have about the same PER as the CBA mice on the egg albumin diet Experiment II clearly indicates a qualitative difference between casein and egg albumin in the NMRI and CBA mice It has previously been shown that casein is inferior as protein food compared to egg albumin in the CBA mice (Munck 1964) The difference in biological value in this respect is most likely to be due to the relatively low content of cysteine and tryptophane in casein For the discussion on obesity which will follow in the other chapters it has to be pointed out that the Swiss mice on the diets used in these two experiments seem to be comparatively more resistent to changes in the protein quality

CHAPTER IV

"Watch out for fads and fakes There is perhaps no field so rife with charlatans as that of providing people with useless substitutes for long term maintenance of dietary discipline which is the key to obesity control

Sebrell 1957

THE EFECT OF A DIET COMPOSED OF THE AVERAGE 1960 CONSUMPTION IN SWEDEN COMPARED WITH A DIET SUGGESTED BY THE NATIONAL INSTITUTE OF PUBLIC HEALTH AND WITH A THIRD OF LOW CARBOHYDRATE AND HIGH FAT CONTENT, ON GROWTH BODY FAT CONTENT AND PROTEIN FAT RATIO IN TWO STRAINS OF MICE

by

STIG LARSSON

In a study by Blix *et al* (1965) a survey was made of the different nutrients consumed by Swedish people from 1925—1964, and the changes in consumption of these during this period The results from this were related to suggestions about a revision of diet from a public health point of view Among the changes in the consumption of different nutrients during these years, an increase of fat intake and a decrease in the use of bread and milk were noted

In several studies it has been found that rats and mice are suitable animals on which to test different diets, particularly when the interest is paid to growth and fat deposition of the body (cf Ericson *et al* 1962*b* and Larsson 1966*a*) In such tests not only growth and food efficiency but also carcass composition with regard to fat, protein and water content are measured The carcass composition as a function of dietary effects is of importance in problems concerned with obesity (Larsson 1966*a*) as well as with meat production in farm animals (*cf* Larsson *et al* 1966)

The present study was performed in order to compare the effect of "1960 year s diet in Sweden with a diet based on the suggested changes (Blix *et al* 1965) by measuring the parameters just mentioned A third diet composed on the basis of suggestions by "food fadists' was also included in the study This diet was poor in carbohydrate but high in fat content

Material and Methods

Twenty male mice of the Swiss and CBA strains were used in each group The animals were 22 days old at the start of the experiment

The main composition of the diets may be seen in Table XI After mixing and grinding the various ingredients the mixture was run through a meat mill The water content of the diets was decreased by transferring the food as thin layers upon baking pans After this the pans were placed in an oven at 35°C with circulating air until the water content decreased to approximately 25 % The diets were then frozen until used

The mice were given the diets *ad libitum* with free access to tap water They were kept individually in macrolon cages (10×16 cm) The weight

of the animals was measured once a week, and the food consumption every day The diets were never kept in the cages for more than 24 h without changing to fresh food

After two weeks on the diets, the mice were killed by decapitation Each animal was then subjected to carcass analysis as described in Chapter I

TABLE XI

The composition of the three diets used in Chapter IV A= 1960 diet B=revised diet and C=high fat low carbohydrate diet

	A	*B*	C
	g	g	g
Vegetables[1])	2 8	4 6	4 0
Fruits and berries	12 5	15 4	15 4
Potatoes	17 1	19 1	2 0
Milk and cheese	29 5	29 6*)	29 6
Meat fish and egg[2])	12 8	10 5 **)	35 5
Bread and other cereal products	14 6	22 3	0
Fat (butter margarine and oils)	3 7	2 2	11 5
Theoretically calculated amount of calories per g food	1 8	1 6	2 1

Cellulose added to give 1 6 cal/g theoretically
[1]) Green vegetables 65 % dried peas and beans 35 %
[2]) Sausages 20 % ox meat 40 % pork 20 % cod 10 % and whole eggs 10 %
*) skim milk
**) trimmed meat no sausages

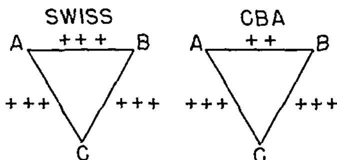
Results

Table XII shows the effect of the three different diets on growth body fat content, protein — fat ratio and body water content in the Swiss and CBA mice It may be seen that there is no difference in growth due to the various diets The body fat content of the CBA mice is lowest in those given diet *B*, and highest in the animals given diet *C* (Table XII and Fig 8) In the Swiss and CBA mice differences are noted between diet *A* and *B* with regard to body fat content Both strains of mice given diet C have a significantly higher fat content than the other two groups (Table XII and Fig 8)

TABLE XII

The effect of the three different diets described in Table XI on growth body fat content protein fat ratio and body water content in male Swiss and CBA mice Mean ± s e m 20 animals in each group Differences in Fig 8

Diet	Growht g in 14 days		Fat content % on dry wt		Protein — fat ratio		Water content %	
	Swiss	CBA	Swiss	CBA	Swiss	CBA	Swiss	CBA
A	10 5	8 7	28 2±1 48	35 2±1 91	1 9	1 6	68 3±1 23	66 9±1 02
B	11 1	8 5	21 3±1 41	28 2±1 81	2 6	2 0	69 2±1 16	68 5±1 11
C	10 8	8 9	36 3±1 87	49 6±2 51	1 4	1 0	61 4±2 01	52 4±2 13



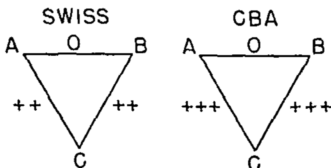


Fig 8 Differences in body fat content and in carcass water of Swiss and CBA mice given three various diets A B and C after values found in Table XII Differences calculated according to Student's t test +++ = p<0 001 ++ = p<0 01 and O = no difference

Table XII also shows that the protein — fat ratios largely follow the body fat content in the different groups The body water contents of the mice from both strains given diet *A* and *B* do not reveal significant differences (Fig 8) In both strains the water contents of those given diet C, however, have significantly decreased values compared with the other groups (Fig 8) No differences in food intake between the animals on the three diets were noted

Discussion

In a recent study on the relation between the total caloric intake and the intake of single nutrients in Swedish food, Blix (1965) found that many groups in Sweden particularly among women did not eat sufficient amounts of calories As a consequence of this finding it was realised that these groups did not consume enough protein The study led to a survey on the total average consumption of various nutrients in Sweden (Blix *et al* 1965) and ended with recommendations for a change in food habits These changes were suggested on the basis of common public health opinion but no experimental studies were made Among the recommendations a reduced fat intake was put forward and an increase in the consumption of skim milk, bread potatoes and vegetables

It has repeatedly been emphasized that the composition of the diet plays an important role for the etiology of obesity in various species (Larsson 1966*a* Larsson *et al* 1966 and Chapter III) It has to be pointed out that the diet composed on the suggestion of Blix *et al* (1965) — diet *B* — gave the lowest body fat content in both strains In the present study the different diets were offered the animals *ad libitum* The strain differences in eating frequencies found in Chapter VII could therefore reflect the results On the other hand it should be mentioned that the effect of the three diets in each strain revealed large differences especially with regard to body fat content and protein — fat ratio of the carcass

The reason why the diet suggested by the Swedish National Institute of Public Health (diet *B*) gives more favourable values regarding low carcass content of fat and high protein — fat ratio than the diet composed of the average consumption in Sweden 1960 (diet *A*) seems to be different participation of fat By decreasing the caloric portion of fat and sugar with corresponding increase in the calories coming from bread milk (as

skim milk) and cheese and recommending trimming of the meat one obtains a diet where the biological value is relatively increased, without cutting down the necessary nutrients minerals and vitamins Diet C contained low amounts of carbohydrates including a decreased starch content due to a cut down in bread, cereals and potatoes The fat content of the diet was comparatively high but within physiological limits The motive for inclusion of diet C in the study is that semi scientific literature has from time to time contained advice such as "calories don t count (Taller 1961) and related ideas such as in "the drinking man s diet (Jameson and Williams 1964) Fenton and Dowling (1953) found that excessive carcass fat deposition was accelerated in some strains of mice by giving a diet high in fat content Fabry *et al* (1964) showed that rats fed *ad libitum* become fatter on a high fat diet than on a carbohydrate diet The present study also shows that diet *C* is without value if a decreased body fat content is desired When comparing the amounts eaten by the mice no significant differences were observed between the different groups In this connection it has to be pointed out that the three diets, at least theoretically were isocaloric due to the dilution with cellulose Further, no essential differences in the growth rates were observed Nevertheless the amount of fat deposited in the body varied significantly depending on the diet given The protein content of the mice of the different groups was essentially the same Marked differences were observed when comparing the body content of water between diet *A* and *B* mice *vs* diet C mice where the water content was exceedingly low As has been shown by previous studies and in this publication, the water content of the body in obesity or in conditions with increased body fat is significantly lower than in other animals (Ericson *et al* 1962, and Larsson 1966*a*) Therefore it is clear that suggestions that a diet for obese individuals should contain high amounts of fat with little or no carbohydrate could be dangerous as obese subjects trying to loose weight according to such ideas would loose nothing but body water which is already in shortage In a study by Ericson *et al* (1962*b*) is was found that degenerative changes in the liver were very closely associated with low water content of this organ Further it was shown that the biological value of the diet protein was directly related to the fat content of the liver (Ericson *et al* 1962*b* and Larsson 1966*a*) In this respect there was also a direct relation between the fat content of the liver and that of the body (Larsson 1966*a*)

The mice given diet C especially from the CBA strain developed obesity — an obesity which could be regarded as nutritionally induced Even if the hereditary participation is obvious between the Swiss and

CBA mice receiving the same diet it is again of importance to stress that merely the type of diet is an etiological factor for obesity Cohn *et al* (1965) have reported that force fed rats or those fed *ad libitum* deposited more fat when given a diet with high fat content free from carbohydrate which is in line with the results found in the present study in mice given diet C

Most studies, however, have been undertaken with diets which could be considered as synthetical and highly concentrated with regard to the caloric content In the present study even if the diets were dried due to the experimental conditions, cellulose was added to diet *A* and *C* It is therefore clear that it was not only the caloric density of the diet that guided the nutritional fate of the mouse but the composition of the food given Even if hereditary factors, also in this instance play a role one thus has to consider external factors such as nutritional in order to produce an obese mouse

The results may indicate that with a relatively constant protein value other nutrients have the ability of causing increased fat deposition

In the present experiments one has to take into account that the diets after mixing were dried at an elevated temperature The biological value of proteins will decrease in diets stored or manufactured at higher temperatures particularly in the presence of reducing substances (*cf* Reimius 1955, Ericson and Larsson 1962 and Larsson 1966*a*) The primary cause for this loss is due to biological unavailability of lysine (Ericson and Larsson 1962 and Larsson 1966*a*) Although one has to consider a decrease in biological value of the diets used in this study, the temperature used when drying the food was only 35°C for a comparatively short period of time Reimius (1955) made a diet composed to correspond with the average food of the Finnish people He noticed that if the dried diet was kept at 140°C for 30—40 minutes the result when giving this diet to rats was a complete cessation of growth presumably due to the lack of biologically available lysine Under practical conditions in humans some of the diet constituents are unprocessed while others are fried or heated with water Thus direct comparison between the results from the present study with humans cannot be made On the other hand it might be assumed that the "strength of the treatment of the food will vary from household to household On the basis of the accumulated data on the effect of foodprocessing it is obvious that the biological value of various diets is probably less than that indicated in tables giving protein content and other data

CHAPTER V

"The simple observation reported herein may serve to demonstrate that seemingly minor portions of an experimental protocol may produce unrecognized effects

Soyka 1966

PRODUCTION OF GOLDTHIOGLUCOSE OBESITY IN DIFFERENT STRAINS OF MICE

by

STIG LARSSON

In studying the LD_{50} dose of goldthioglucose Brecher and Waxler (1949) found that a single intraperitoneal injection of the substance — 1 mg per g body weight — resulted in a high percentage of hyperphagia in the surviving mice finally leading to overweight and obesity This observation initiated many studies on the nature and origin of this obesity Thus Marshall Barrnett and Mayer (1955) found that goldthioglucose produced specific lesions in the ventromedial parts of the hypothalamus that is the structures known to contain the satiety part of the feeding center (*cf* Brobeck 1955) Later studies have shown that goldthioglucose selectively accumulates in these parts of the brain (Liebelt and Perry 1957, Liebelt et *al* 1960 Debons et *al* 1962 Swartz, Christian and Andrews 1960 and Edelman *et al* 1965)

It has also been demonstrated that different strains of mice evidently do not have the same sensitivity to goldthioglucose with regard to the development of obesity (Liebelt *et al* 1960, and Larsson 1966*b*) In the course of the present study goldthioglucose obesity was chosen for the following reasons It was felt that this type of obesity was suitable for comparison with the nutritional obesity produced by certain foods Further the various strains of mice used in the present investigation have shown definite strain pecularities and the preliminary study (Larsson 1966*b*) revealed these differences with regard to the reaction to goldthioglucose

Material and Methods

Male mice of the Swiss NMRI and CBA strains were used They were fed *ad libitum* on the standard laboratory diet described in Chapter I and were given free access to tap water

The mice were kept individually in the small sized (10×16 cm) macrolon cages

Goldthioglucose as a 20 % oil suspension (Solganol B oleosum Schering) was administered intraperationeally to the mice in the fed state or after 24 hours without food The age of the animals when injected was 30—35 days In connection with the injections of goldthioglucose

blood samples were taken and analyzed for glucose as described in Chapter I

The weight of the animals was measured once a week

Results

Table XIII indicates that a dose of goldthioglucose higher than 1 mg per g body weight given to CBA mice starved for 24 hours results in 100 % mortality, while the same amount given to starved Swiss and NMRI mice of the same age caused a mortality of only 10 % The LD_{50}-dose in these two strains was found to be about 2 0 mg per g body weight

TABLE XIII

Mortality of Swiss NMRI and CBA mice injected with different doses of goldthioglucose S=mice starved for 24 h F=fed mice n=number of animals

		Mortality in %			
Amount of goldthio glucose injected (m/g body wt)		0 5 n=50	1 0 n=75	1.5 n=55	2 0 n=60
Strain					
Swiss	S	0	10	30	50
	F	20	90	100	
NMRI	S	0	10	20	50
	F	20	80	90	
CBA	S	40	80	100	
	F	100			

Table XIV A gives the incidence of obesity — calculated from the relation between the amount of body fat and the fat free body weight (Figure 1 Chapter I) — in surviving mice 60 days after the injection of goldthioglucose It may be noted that with injection of 1 0 mg per g body weight the incidence of obesity is highest in the CBA strain Table XIV B indicates that the frequency of overweight follows the frequency of obesity only in the CBA mice whereas in the Swiss strain, overweight after the administration of goldthioglucose is found only when the highest dose is given In the NMRI mice a certain difference between the incidence of obesity and overweight is also noted

TABLE XIV A

Frequency of obesity (see Fig 1) in % of surviving animals of the Swiss NMRI and CBA mice Starved animals

Strain	Dosage of goldthioglucose (mg/g body wt)			
	0 5	1 0	1 5	2 0
Swiss	5	30	40	50
NMRI	5	35	45	60
CBA	30	70		

TABLE XIV B

Frequency of mice with overweight (increased body weight compared with controls) in % surviving animals Starved animals

Strain	Dosage of goldthioglucose (mg/g body wt)			
	0 5	1 0	1 5	2 0
Swiss	0	0	0	15
NMRI	0	20	40	50
CBA	30	70		

The blood sugar values (in mg per 100 ml blood) in the three different strains after 24 hours starvation were in the Swiss mice $47 \pm 2\ 5$, in the NMRI $82 \pm 5\ 8$ and in the CBA mice $85 \pm 5\ 7$ (the difference between the Swiss mice and the two other strains being statistically significant $p < 0\ 001$)

When goldthioglucose was injected into fed mice, a considerable increase in mortality was noted (Table XIII) On the other hand it was found that the incidence of obesity increased in the surviving animals

Discussion

In a preliminary study it was found that strain differences existed with regard to the development of obesity after a single injection of goldthio glucose (Larsson 1966*b*) Liebelt *et al* (1960) showed that gain in body weight and mortality in mice following a single injection of goldthioglucose was dependent on the strain and the dosage They also found a parallel response to the effects of goldthioglucose with regard to mor

tality and weight gain of CBA mice compared with the other strains studied This was supported by the present study, and also shown to be the case with the final body fat content It was also of interest to note that in general the data from the present study is in accordance with the findings of Liebelt *et al* (1960) suggesting that sublines of this inbred strain in another laboratory respond similarily

The strain differences with regard to goldthioglucose in the present study related to those reported in the previous chapters seem to indicate that goldthioglucose is not only a suitable substance with which to produce obesity, but is also a substance which could be used for comparing different hereditary conditioned characteristics of other factors involved in causing obesity

A 24 hour fast period preceeded the injection of goldthioglucose in half of the animals under test Determinations of blood sugar in these mice revealed that the Swiss variety had the lowest levels after the period of starvation (see also Larsson 1966*b* and Chapter II) It is possible that the resistance to goldthioglucose, both with regard to toxicity and the development of obesity, is partly due to the extreme fall in blood sugar in this strain It has been reported that feeding the animals before the injection of goldthioglucose diminishes the incidence of permanent damage and hyperphagia by 30—50 per cent (*cf* Anliker and Mayer 1957) a result which could not be verified in the present investigation Also in a study by Soyka (1966) it was found that the lethal effects of gold thioglucose were elevated in mice given glucose simultaneously with the injection of goldthioglucose As the mortality increased, the incidence of obesity as judged from the body weight also increased This finding on the relation between the mortality and the incidence of obesity is confirmed by the present study Soyka (1966) also showed that the administration of insulin decreased the mortality and the incidence of overweight when goldthioglucose was given One explanation for these findings could be the blood sugar level *per se* Edelman *et al* (1965) studied using various techniques, the accumulation of gold in the hypothalamus and the development of hyperphagia and obesity, in mice given goldthioglucose intravenously at different blood glucose concentrations They found that the gain in body weight the extent of the hypothalamic involvement and the degree of hypothalamic gold accumulation generally reflect the blood glucose concentration produced in the first few minutes following injection The same authors presented a hypothesis correlating the blood glucose concentration with the degree of appetite inhibition Hence the degree of appetite inhibition of the ventromedial area should be proportional

to the number of nerve cells activated The generation of the action potential of the satiety neurones should also be dependent on the metabolism of a specific quantity of glucose in the glial cells The hypothesis is confirmed by various biochemical and neurophysiological studies Thus, differences in the incorporation of radioactive phosphorus into ATP and creatine-phosphate fractions as well as differences between the absolute amounts of these fractions in the hypothalamic areas in hungry and fed rats, emphasizes the specificity in the behaviour of the hypothalamic region (Forssberg and Larsson 1954 and 1955) Biochemical characteristics of the hypothalamus with regard to the fate of glucose have been found (Chain Larsson and Pocchiari 1960), as have differences during hunger and satiety (Larsson 1967) The electrical activity of the hypothalamic region has also been found to be related to changes in the blood chemistry (Anand Dua and Singh 1961 and Anand, Chhina and Singh 1962) and by the administration of amphetamine (Brobeck, Larsson and Reyes 1956)

In the present study as previously mentioned, the incidence of obesity was lowest in the starved Swiss mice where the blood sugar values after a 24 hour fast were lowest Preliminary studies where goldthioglucose was given to alloxan diabetic mice suggest, however, that this type of diabetic state with lack of insulin will contribute towards affording protection against goldthioglucose (Rerup and Larsson, unpublished) Thus it has been found that the injection of goldthioglucose in the alloxan diabetic mice will decrease the toxicity of this substance in spite of the exceptionally high levels of blood sugar As mentioned by Soyka (1966) and further supported by the present study and by preliminary results obtained by Rerup and Larsson (to be published) it is a little surprising that the reaction towards a toxic substance can be altered by more or less physiological or endocrinological changes in the internal environment Because of this goldthioglucose injected in smaller doses might prove to be a useful tool in differentiating between different types of obesity

In line with the present opinion are the findings by Liebelt and Perry (1957) who noticed striking differences between two strains of mice regarding the susceptibility to goldthioglucose obesity They explained this as being due to the varying sensitivity of the food intake — regulating mechanism to the toxic effect of goldthioglucose

It is of interest to note the observations made by Oro Wallenberg and Bolme (1965) on electrical stimulation of certain areas of the diencephalon and mesencephalon resulting in elevated levels of plasma FFA and glycerol both being indications of increased lipolytic activity

Goldthioglucose injection results, among other things in an accumulation of gold in the diencephalon causing brain lesions (Swartz *et al* 1960) The areas damaged by the goldthioglucose are associated with the regulation of food intake (Marshall *et al* 1955)

In the present study it was found that the development of overweight and obesity was associated with a decreased tendency to mobilize fat In this connection it is of interest to note that the mice obese from the injection of goldthioglucose may have up to 100 times or more circulating insulin in the blood (Rerup and Larsson to be published) which may explain their decreased ability to mobilize fat This tendency was particularly striking in the Swiss mice injected with goldthioglucose, which resulted in an elevated fat content of the body, without overweight

With the accumulated data including previous and present results, it is tempting to suggest that the effect of goldthioglucose as a cause of obesity is dependent upon several factors It seems that the toxicity and the occurence of obesity is increased in the fed state (*cf* Edelman *et al* 1965 and the present study) The accumulation of gold in the ventromedial cells of the hypothalamus seems dependent on the glucose level of the blood As judged from the experiments by Edelman *et al* (1965) the accumulation of gold in the ventromedial nucleus is directly proportional to the blood glucose level, as well as to the percentage of overweight In the present study is was found that the incidence of obesity as judged from increased body fat content was higher in mice injected with goldthioglucose in the fed state On the other hand mice made hyperglycaemic with alloxan injections seem to be less sensitive to the effects of goldthioglucose The injection of this substance in 24 h starved alloxan-diabetic mice resulted in a significantly higher incidence of death than in the fed state (Rerup and Larsson to be published) Therefore it could be suggested that the incidence of mortality depends on the levels of biologically active insulin Soyka (1966) found that hypoglycaemia caused by insulin decreased the mortality and the incidence of obesity in the goldthioglucose injected mice Earlier Drachman and Tepperman (1954) found that fasting prior to the injection of goldthioglucose increased the survival rate in the mice Further it was found that adaption of the animals to a high fat diet prior to injection markedly enhanced the toxicity of goldthioglucose Whatever the explanation for the various reactions towards goldthioglucose may be, there is reason to suggest that the blood glucose level *per se* is not the primary cause

CHAPTER VI

"A great deal of nonsense has been talked about this subject, and textbook after textbook has repeated the fallacy that almost unattainable levels of exercise are required to burn a pound of fat'

Kennedy 1964

THE EFFECT OF MUSCULAR ACTIVITY ON FAT MOBILIZATION AND BODY FAT CONTENT IN NORMAL AND OBESE MICE

by

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Previous studies have demonstrated the importance of muscular exercise as a means of reducing weight in goldthioglucose obese mice (cf Mayer 1955, and Larsson and Strom 1957) None of these studies, however included body fat determinations As has been pointed out earlier in this study, overweight and obesity are not necessarily the same condition and therefore the only safe method of studying factors that may influence obesity is to measure the body fat content Parizkova and Stankova (1964) found that in normal rats muscular exercise resulted in a lower fat deposition

It has been shown in the preceding chapters that the mice exhibit strain characteristics towards some factors of importance for the etiology of obesity It could therefore be possible that the effect of exercise should differ according to the strain used both in normal and obese mice It was felt necessary that the exercise ought to be studied not only for its effects on the body weight but more important for its effects on the fat content of the body food consumption and fat mobilization

Material and Methods

In this chapter only male mice have been used Normal female rats and mice have an oestrus behaviour which is accompanied by an increase in spontaneous activity Brobeck, Wheatland and Strominger (1947) have shown that the food intake is depressed during oestrus, leading to a weight loss while the opposite events take place during dioestrus

Therefore in this investigation where other factors influencing food intake and fat deposition are studied it was felt that male mice were more suitable

Swiss NMRI and CBA mice were used They were fed *ad libitum* on the diet described in Chapter I and had free access to tap water

Depending on the experimental conditions they were weighed once or twice a week

After the experimental period the animals were killed Part of the epididymal fat pad was usually removed and the net release of glycerol and FFA determined *in vitro* as described in Chapter I The carcass content of fat water and protein was determined according to Chapter I

Fig 9 The arrangement for forced activity in the mice A=boxes where the animals are running, B=running carpet

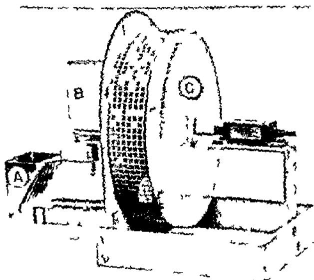


Fig 10 The arrangement for spontaneous activity in the mice A=feeding box B=water supply and C=rotating wheel

Forced activity Mice of the Swiss, NMRI and CBA strains were subjected to a training regimen in a treadmill (Fig 9) The animals were gradually trained to run on the moving carpet at a speed of 25 cm/sec The experimental period lasted for 30 days The mice were running for 60 minutes in 6 consecutive 10 min periods where 2 min periods of rest were superimposed When not in the treadmill the animals were kept individually *in the small type of macrolon cages (10×16 cm) as did the* controls The food intake was measured daily

Goldthioglucose obesity was produced as described in Chapter I

Spontaneous activity The running activity of normal and goldthioglucose obese mice of the Swiss NMRI and CBA strains was measured The apparatus may be seen in Figure 10 The animals were observed for periods of 30 days

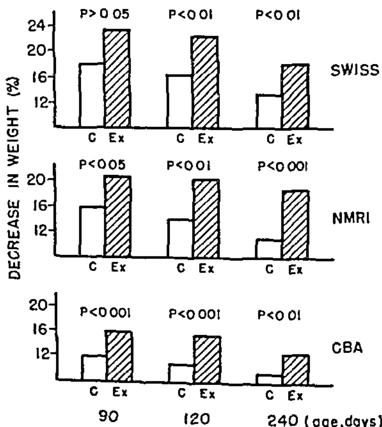


Fig 11 The effect of exercise in Swiss NMRI and CBA mice on decrease in body weight after 4 h *of fast compared* to non exercised fasted animals The decrease in weight in %

Influence of cage size Normal mice of the Swiss, NMRI and CBA mice were kept individually in either the smallest sized cages (10×16 cm) or in the largest sized (23×39 cm) for 60 days after weaning after which the carcass analysis was performed All animals were given the standard laboratory diet *ad libitum*

Groups of exercised mice were starved for 24 h and the decrease in weight recorded

Results

Forced activity Table XV shows the effect of forced exercise in normal mice of the Swiss NMRI and CBA strains It may be seen that the NMRI mice did not change in weight on forced activity compared with the controls, while both the Swiss and CBA mice had a negative weight change The same table also indicates that the Swiss mice had the same food intake over the 30 day period whether they were forced to run or not Both the exercised NMRI and CBA mice had significantly increased food intakes compared with the unexercised controls After the period of forced activity the NMRI and CBA animals had significantly lower fat contents The muscular activity caused an increase in the protein — fat ratio in all the strains studied

As shown in Table XVI forced activity in all the three strains resulted in higher net release of glycerol and FFA from the adipose tissue *in vitro* The response to adrenalin *in vitro* did not change from the controls

TABLE XV

The effect of forced activity (1 h per day for 30 days) in normal male Swiss NMRI and CBA mice Age of the animals at the start of the experiment was 90 days Ten animals used in each group The changes are related to the values in unexercised controls Differences calculated according to Student s t test a)= $p<0\ 001$ b)= $p<0\ 01$

Strain	Weight change g	Change in food intake g	Change in body fat content %	Change in protein/ fat ratio %
Swiss	—5 9)	—4	—15	+3 a)
NMRI	—0 6	+55	—23 b)	+79)
CBA	—2 0 b)	+43	—30 b)	+81)

TABLE XVI

The change in n t rele of glycerol and FFA from adipose tissue *in vitro* after forced activity in Swiss NMRI and CBA mice Differences calculated according to Students t test a)=$p<0.001$ b)=$p<0.01$

Strain	Change in net release from adipose tissue in % of values of controls	
	Glycerol	FFA
Swiss	+25[b)]	+36[a)]
NMRI	+46[a)]	+51[a)]
CBA	+46[a)]	+62[a)]

Spontaneous activity Table XVII shows the effect of spontaneous activity followed to 30 days in obese male mice (from 30 days of age) of the Swiss NMRI and CBA strains It may be noted that the weight gain of the "controls" is more than twice that of exercised mice in general The same Table shows that the fat content of the body in all strains after activity is significantly lower than in the controls and that in obese animals the carcass fat content has gone down to the value of normal animals The protein — fat ratio in these animals also correspond to that of the non obese mice

TABLE XVII

The effect of spontaneous activity in squirrel wheels on weight and body fat content in obese Swiss NMRI and CBA mice compared with unexercised animals Differences calculated according to Students t test a)=$p<0.001$

Strain	Weight change %	Change in body fat content %
Swiss	—80[a)]	—42[a)]
NMRI	—55[a)]	—68[a)]
CBA	—82[a)]	—85[a)]

Influence of the cage size Table XVIII shows that the animals kept in the small cages have a significantly higher body fat content

Figure 11 shows that exercised animals decrease more in weight on starvation than un exercised controls

TABLE XVIII

The effect of the cage size in normal Swiss NMRI and CBA mice on the body fat content Mean±s e m 30 animals in each group Differences calculated according to Students t test a)=$p<0.001$ b)=$p<0.01$

	NMRI	Swiss	CBA
Large cage	26 3±1.33[b)]	21 4±1 73[a)]	30 7±1 42[b)]
Small cage	31 3±1 00	29 5±1 34	36 4±1 27

Discussion

The results from this study indicate that the muscular exercise will decrease the fat content of the body with or without a weight change of the body

The effectiveness of exercise in the treatment of obesity has been discussed earlier (*cf* Mayer 1955) but surprisingly little practical application has been made This depends no doubt upon the experimental set up If experimental animals are exercised or human beings subjected to muscular activity, observations have been made that the body weight often shows little or no decrease This has lead to the conclusion that exercise is of minor importance in the treatment of obesity This could of course be true under certain circumstances if one considers obesity and body weight as primary dependent upon each other As has been discussed elsewhere in this study, obesity is not always followed by an increased body weight but should rather be recognized when the fat content of the body is higher than normal

Judging from the results on the effect of exercise in mice where the animals are starved for 24 hours (Fig 11) it is evident, that all animals, regardless of strain and age will decrease more in weight than corresponding non exercised mice without food This simple finding suggests that the exercised animals have a higher ability to mobilize fat In view of the findings from the *in vitro* studies this is not surprising since the fat mobilization as measured by the net release of glycerol and FFA is increased in the exercised animals Mayer (1955) has pointed out that exercise entail a considerable expenditure of energy It is difficult to understand why muscular exercise has received such a negligable attention in many studies where the interest has been focused on the causes and treatment of obesity One of the arguments used, is that muscular activity requires little caloric expenditure when performed moderately An other argument is that physical activity is accompanied by an increased food intake which would cover the extra calories utilized Still, as pointed out by Parizkova (1966) the increased energy output due to enhanced physical activity in sportmen may require correspondingly increased caloric input reaching 7000—8000 kcal per day In these people the high intake of calories will lead to a high ratio of lean body mass at the expense of fat. Miller and Mumford (1966) have discussed the metabolic situation in humans subjected to moderate exercise They give evidence for the view that the extra energy output *per se* during exercise may not be the important factor as pointed out by Mayer (1955) but rather the las-

increase in metabolic rate following upon muscular activity Exercise or electrical stimulation of the skeletal muscles results in increased fatty acid utilization in muscles (George 1964, and George and Vallyathan 1964) Further, as shown by Parizkova (1966) the amount of FFA released *in vitro* from the epididymal adipose tissue is higher in running animals an observation which can be confirmed by the present study Increased sensitivity to adrenaline with regard to the release of FFA has also been noted after exercise (Parizkova 1966)

From a physiological point of view the present results seem to indicate that exercise is of fundamental importance in relation to the fat content of the body as well as to the fat mobilizing capacity In this connection Oro *et al* (1965) have given indirect support in studies on the effect of diencephalic and mesencephalic stimulation in anaestetized dogs on the release of FFA and glycerol In these brain parts the localization of functions related to locomotor activity as well as autonomic functions such as increased heart rate, blood pressure and muscular blood flow are found (Rushmer *et al* 1961) Studies in experimental animals suggest a close integrative relation between the central areas regulating food intake and locomotor activity (*cf* Kennedy 1964) Thus when lesions are produced in the ventromedial parts of the hypothalamus the resulting hyperphagia is always accompanied by decreased spontaneous activity

It can be discussed whether the "normal laboratory conditions for mice and for other experimental animals are physiological in a true sense The animals are usually kept in comparatively small cages with little opportunity to move freely Normally all animals exhibit a certain degree of spontaneous locomotor activity When this opportunity is lost or reduced it has been shown that the immobilization may lead to obesity (Ingle 1949) Mayer *et al* (1954) have suggested that a non responsive range exists with regard to change in food intake on both sides of the usual zone of activity The present experiments confirm these findings by demonstrating that depending upon the size of the cage the fat content of the body will vary regardless of the strain of mice used

Tepperman and Tepperman (1964) have introduced the term adaptive hyperlipogenesis from a practical point of view The same authors have later applied and extended this term and found it be valid for various forms of experimental obesity (*cf* Tepperman and Tepperman 1965) Without taking into the discussion the adequate intermediate stimulus it seems logical to include muscular inactivity into the term adaptive hyperlipogenesis since inactivity, as found in the present study always

leads to increased body fat deposition and a decreased ability to mobilize fat in the fed state as well as after various periods of food deprivation

The energy cost of exercise is approximately proportional to body weight From this follows that an over weight individual will require more energy for the same amount of work than would a lean individual In the present study forced activity in obese mice proved to be effective not only with regard to weight reduction but in particular to decrease the body fat content This was also true for non obese exercised animals Hypothalamic obesity in the rat and mouse is characterized by a considerable degree of inactivity (*cf* Kennedy 1964 and Mayer *et al* 1954) This evidently is depending on the extent of the hypothalamic lesion as it seems as the same areas of the hypothalamic region is also gouverning the locomotor activity (*cf* Kennedy 1964) The obesity caused by goldthioglucose only partly follows the pattern of hypothalamic obesity As previously shown and confirmed by the present experiments the gold thioglucose obese mice are primarily inactive when placed in a squirrel wheel (Mayer 1964 and Larsson 1957) As demonstrated previously and confirmed by the present study the obese mice gradually increase the spontaneous activity so that they loose weight and body fat Mayer (1955) found that the spontaneous activity in goldthioglucose obese mice is not impaired Mayer *et al* (1954) have suggested that within the so called sedentary range the hunger-satiety mechanism is not working properly Thus within this range the food intake of animals is partly exceeding the actual caloric needs By exercise the sedentary range is avoided which in normal individuals will cause the regulatory mechanism to act more accurately An example of this found in this chapter, is the observation that the size of the cage is of importance for the extent of fat deposition of the body It has also been reported that exercise up to about 6 hours per day in rats will result in increased food intake but no weight change (*cf* Mayer 1955) In the present study no attempts were made with longer periods of forced exercise than one hour The CBA and NMRI mice subjected to this mild type of exercise responded with a significant increase in food intake In spite of the increased food intake the CBA mice lost weight while no weight change was observed in the NMRI mice The Swiss mice subjected to forced activity did not compensate the extra caloric expenditure with increase in food intake As a natural result of this the animals lost weight However in all the animals the forced activity decreased the body fat content In the CBA mice roughly 50 % of the total body fat disappeared after 1 hour of forced activity daily for 30 days Still in this strain the increase [illegible]

was about 40 % more than the unexercised controls kept in small cages The relative weight loss, however, was only about 10 % compared to the controls It thus seems justified to state that under the experimental conditions described exercise is an effective factor for weight control and which is more important, as a regulator for the fat content of the body It has also to be pointed out that strain differences exist, but the general trend is not changed by this fact

The present chapter shows that when studying problems concerned with body fat content, lipogenesis and lipid mobilization in relation to obesity, the simple determinations of body fat weight and food intake are enough to get information of the net effects on the organism To some extent, however, it is of interest to study some of the "local parameters for example in the adipose tissue Friedberg *et al* (1960) have found that acute exercise lowers the plasma FFA content followed by a significant rise in plasma FFA concentration immediately after exercise The rise in FFA concentration after exercise indirectly points towards an increased mobilization of fat Further, Fritz *et al* (1958) studied the fatty acid oxidation by skeletal muscle during rest and activity *in vitro* and found that stimulated muscle utilized twice as much fatty acids as corresponding muscle at rest The present study indicates that the exercised animals have a higher net release of glycerol and FFA *in vitro* from adipose tissue Preliminary experiments seem to indicate that the muscles from exercised mice utilize more fatty acids than the unexercised muscle even without stimulation All experiments in the same line seem to indicate that exercise not only will facilitate the adipose tissue to a higher fat mobilizing capacity but also facilitate the muscle to utilize more fatty acids even at rest

Regarding the animals living in small cages with little opportunity to exercise spontaneously it is evident that the sedentary range is a border line in nutritional homeostasis which deserves much attention Thus, it can be stated that muscular inactivity is an important factor for the etiology of obesity

CHAPTER VII

"Nearly all of the published studies of the regulation of food intake neglect the fact that the total amount of food eaten is always the product of two factors, the number of meals multiplied by the intake of the average meal

Brobeck 1955

EATING BEHAVIOUR IN NORMAL AND OBESE SWISS NMRI AND CBA MICE

by

STIG LARSSON

It has been demonstrated in several investigations that the consumption of the daily food ration within short periods considerably increases lipogenesis in rats (Tepperman *et al* 1943, and Hollifield and Parson 1962) Attempts have been made to repeat these experiments in mice, but without success probably due to the higher metabolic rate in these animals which causes difficulties in maintaining the body weight by too infrequent feedings (Larsson, unpublished) In a previous study it was shown that goldthioglucose obese ASw mice ate larger meals less frequently than non obese controls (Larsson and Strom 1957)

In view of other strain differences found in this study, it was of interest to measure the eating frequency in the three types of mice used in this investigation, both in lean and obese animals

Material and Methods

Male mice of the Swiss, NMRI and CBA strains were used The age of the animals at the start of the experiment was 150 days Goldthioglucose obesity was produced as described elsewhere (Chapter I) and the compound injected when the mice were 45 days old At the start of the experiment the obese mice had reached the static phase of obesity

To record the meal frequency the same apparatus as previously described was utilized (Larsson and Strom 1957) The observation time for each mouse in the apparatus was about 14 days The food used was the regular standard food described in Chapter I, formed into small tablets fitting in the feeding apparatus Because of technical reasons about 15 % adhesive material in form of *sacch lactis* was added

Results

Table XIX and figure 12 indicate that normal Swiss mice ate more frequently than did the NMRI mice which in turn had a higher meal frequency than the CBA mice The goldthioglucose obese mice all ate less

frecuently than the corresponding lean litter mates (Table XIX) The obese NMRI and CBA animals ate significantly less frequently than the obese Swiss mice

Figure 12 shows the differences between the various strains indicated by the same system as previously used by Munkner (1963) for other para meters As the food intake did not differ significantly between the lean and fat mice the size of each meal in the obese animals was much larger than in the non obese litter mates

TABLE XIX

Eating frequency in 24 h±s e m in normal and goldthioglucose obese male Swiss NMRI and CBA mice studied for two weeks in the feeding apparatus Differences between lean and obes mice from the same strain calculated according to Student s t test Number of animals in each strain 15 normal and 10 obese a)=p<0 001

Strain	Eating frequency in 24 h	
	Normal	Obese
Swiss	39±1 0)	18±0 8
NMRI	27±0 7)	12±0 8
CBA	19±0 8)	10±1 1

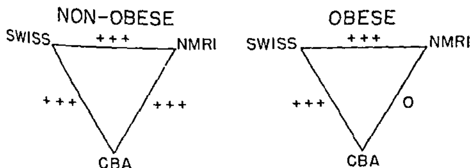


Fig 12 Difference in eating frequen y between normal and obese Swiss NMRI and CBA mice, calculated after the values given in Table XIX according to Student s t test +++=p<0 001 and 0=no difference

Discussion

When studying the regulation of food intake and the factors of importance for the etiology of obesity such as various nutritional strain characteri stics in normal and obese mice it is obviously important not only to

measure the total food consumption but also to record the eating behaviour in the animals over an extended period of time.

In a previous study it was revealed that goldthioglucose obese mice of the ASw strain ate larger meals of food less frequently compared with their corresponding lean litter mates (Larsson and Strom 1957). In the present study this observation was confirmed in all three strains. It was however also shown that particular strain characteristics existed with regard to the feeding frequency. Thus, the Swiss mice, exhibiting many indications of "lean" mice, ate significantly more frequently than the other strains. It is possible that this phenomenon, as such contributes to the lower body fat content generally found in this strain compared with the NMRI and CBA mice. It has been demonstrated that rats can be trained to eat sufficient amounts of food in one hour per day, and studies of the respiratory quotients of these animals indicate increased lipogenesis (Tepperman *et al* 1943). Hollifield and Parson (1962) have later shown that adaptive changes occur in adipose tissue in rats fed for only two hours per day. Thus in these animals the FFA content fell as the rate of lipogenesis increased. Other studies have further revealed that the total body metabolism is markedly altered by various eating habits such as changes in eating frequencies "meal eating *vs* nibbling" (Cohn and Joseph 1960). Similar studies have suggested that less frequent eating but larger meals might lead to obesity. Van Putten, van Bekkum and Querido (1955) found that when rats with hypothalamic hyperphagia were pair fed against control animals, the hyperphagic animals ate the daily ration in a short time but the control rats needed twenty four hours to eat their diet. The hypephagic rats contained about twice the amount of fat but less protein than the control rats. The present results are largely in the same line as those quoted above as it seems that those mice eating less frequently also have the highest body fat content.

Further evidence for the importance of the eating frequency for fat deposition is the finding by Feldman *et al* (1957) that male chickens made aphagic with diencephalic lesions and therefore tube fed developed grossly increased quantities of subcutaneous fat.

The present experiments again indicate the importance of taking into consideration the strain characteristics when studying nutritional problems. From a practical point of view, hereditary factors in obesity must be considered. In clinical studies this has not always been the case. Rather existing genetic basic characteristics are often regarded as psychological factors because of lack of compiling data from experimental animals with clinical conditions in humans.

In the literature very few studies are found concerned with the importance of eating frequencies for the final body composition Anliker and Mayer (1957) found certain differences between the feeding behaviour in various types of obesity They used the Skinner box to observe the meal frequency This apparatus works so that the animal has to press a bar in order to receive the food possibly in reward In the present study, as has been explained previously (Larsson and Strom 1957), the food is available in the cage the whole time which avoids psychological interference as to the question whether the animals have to work for the food or not

In the present study the constancy of the meal frequency within the respective strains is surprising and might be taken as evidence for the strength of the genetic impact on the nutritional state Then as shown in Chapter I, III, IV and VI, external factors such as food composition and muscular activity are of importance in altering and modifying the hereditary destiny

As changes in the dietary pattern can induce significant metabolic alterations (*cf* Tepperman and Tepperman 1965) it is not surprising that studies in meal fed and nibbling rats reveal that the metabolism of the adipose tissue in the meal eating animals is characterized by a marked increase in those metabolic parameters leading to the synthesis and deposition of carbohydrate and fat (Leveille and Hanson 1965) The obese mice of all the strains studied have significantly lower feeding frequency than their non obese litter mates As the obese mice used had reached the static phase of obesity, i e not gaining further weight or body fat content it is possible that the meal frequency is of importance to pattern the machinery of the fat cell in the obese animals towards enhanced lipogenesis or to maintain it at this state There is good evidence for such an adaptation in meal eating animals (Cohn and Joseph 1959 and Leveille and Hanson 1965) It is of course impossible to state whether the differences in the eating behaviour are the cause of the increased body fat content in the mice eating less frequently or if this phenomenon runs parallel to the high body fat content

CHAPTER VIII

"Most dietary experiments imply the assumption that the animal consumes as much of the available food mixture as will improve the animal's nutritive status. Trust is placed in paired feeding as the sole control procedure

Adolph 1947

FOOD PREFERENCES IN DIFFERENT STRAINS OF NORMAL AND OBESE MICE

by

STIG LARSSON

In a previous study it was found that mice of the ASw strain, made obese with goldthioglucose usually gained weight when the food was changed from the ordinary laboratory diet to a free selection of three different diets (Larsson 1957) While gaining weight the mice in general preferred to select a diet with a high fat content The normal mice did not change significantly in weight on free selection

It has been found that the qualitative content of the food is of importance for the development of obesity (Lundbaek and Stevenson 1947 and Strominger Brobeck and Cort 1953) The present results also provide evidence for this observation

Fenton and Dowling (1953) have produced evidence for the view that different strains of mice react differently with respect to body weight when offered a diet with a high fat content The present investigation has also revealed that both hereditary and nutritional factors are important for changes in body composition which may lead to obesity

The present experiments deal with the free selecting of diets by the three strains of mice used throughout this investigation in order to explore whether differences exist in the preferences of mice for the various diets To make this study more complete free selection in experimentally induced obesity (with goldthioglucose) was also measured Besides for the food preference and growth determinations the carcass content of water protein and fat was also measured

Material and Methods

Male mice of the Swiss NMRI and CBA strains were used in this study All mice were fed *ad libitum* with free access to tap water They were given the standard food described in Chapter I until 120 days of age when they were given a free choice of three different diets Table XX gives the composition of the diets The mice on free selection were kept individually in macrolon cages (20×26 cm) and the different diets given as may be see in Figure 13 The corresponding controls were placed in the same sized cages and received the standard diet throughout their lives Goldthioglucose obesity was produced as indicated in Chapter I The

obese animals were not used until they reached the static phase of obesity This is the reason why all animals were fairly old (120 days) when used in the actual experiment

TABLE XX

The composition of the three diets used in the free selection study

Content in g	Diet A	Diet B	Diet C
Egg albumin	250	700	220
Sugar	680	230	220
Corn oil	30	30	60
Lard			460
Premix A)	20	20	20
Premix B*)	20	20	20

*) *Premix A* had the following composition and contained per g vit B 0 25 mg nicotylamide 1 00 mg folic acid 0 01 mg cal ium pantothenate 1 00 mg vit A 2 10 mg (325 IU/mg) vit D 0.225 mg (300 IU/mg) vit 1.25 mg and brewer s yeast up to 1 g

Premix B contained per g sodium chloride 200 mg brewer s yeast 300 mg dicalcium phosphate 5?0 mg cobolt sulphate 2 5 μg manganese sulphate 0 5 μg ferrosulphate 1 65 mg choline bitartrate 2 00 mg and magnesium sulphate 5 00 mg

The food intake was measured daily and the body weight once a week As a certain incidence of diabetes was observed in the obese mice in a previous study on free selection (Larsson 1957) the urine was frequently checked with "Clinistix (Ames) for the occurence of glucosuria

After 30 days on free selection the animals were killed and the carcass content of water protein and fat was measured as described in Chapter I

Results

As may be seen from Figure 14 there is a difference in the selection of diets in the non-obese mice depending upon the strain The NMRI mice seem to prefer a diet lower in fat content than the Swiss and CBA mice The non obese Swiss and CBA mice select a diet which in average contains more than 50 caloric % of fat

The obese mice regardless of the strain have a higher fat intake than their respective non obese litter mates (Fig 14) Between the obese mice

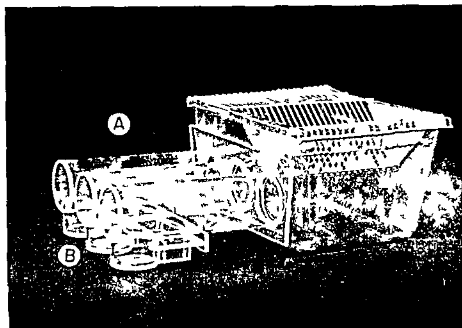


Fig 13 The arrangement used for the free selection studies A=funnels leading to the food cups=B

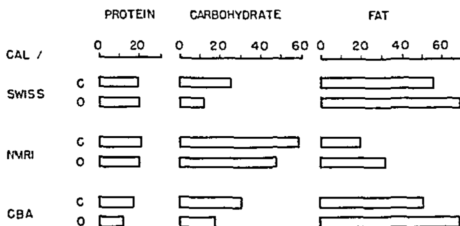


Fig 14 The selection of food in normal (c) and obese (o) Swiss NMRI and CBA mice expressed as caloric participation of protein carbohydrate and fat in %

of the various strains the selection of food is different, the obese Swiss and CBA mice having the highest fat intake The obese NMRI mice gradually decrease the fat intake with corresponding increase in the consumption of carbohydrate (Fig 15) In the obese CBA mice with a high fat intake of about 70 calorie % no decrease with time was noted (Fig 15) Obese Swiss mice select a diet with high fat content for a period of roughly 18 days after which the fat consumption decreases

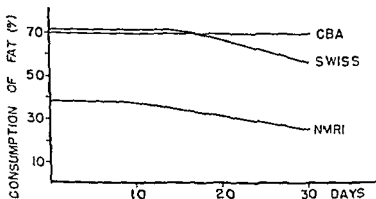


Fig 15 The caloric participation of fat in the food in % in obese Swiss NMRI and CBA mice in relation to time

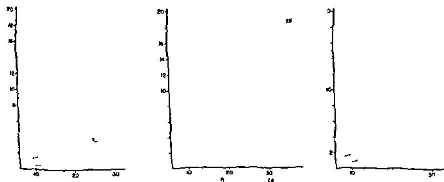


Fig 16 The body fat plotted against the fat free weight of Swiss NMRI and CBA mice on 30 days of free selection of food ⅄=normal mice on free selection ●=obese mice on free selection and O=obese mice on the standard laboratory diet As a comparison the broken lines indicate the limit values for normal mice on the standard laboratory food as given in Fig 1

Figure 16 shows that the body fat content of the animals on free selection always was higher than in the corresponding mice on the standard laboratory diet It may be seen that the difference is particularly large in the NMRI and CBA mice As a consequence of the increased body fat content the protein — fat ratio in the carcass will decrease

Discussion

Largely the results obtained earlier on free selection of diets in obese ASw mice have been confirmed (Larsson 1957) All obese mice consumed significantly more fat than corresponding lean litter mates The obese Swiss and CBA mice had the highest relative fat intake This is in line with the other observations in the CBA strain obtained in the present publication Thus the CBA mice have a comparatively high body fat content on the standard laboratory diet and a comparatively low release of glycerol and FFA from the adipose tissue *in vitro* This strain has also been found to be very sensitive to the composition of the diet and the quality of the protein in the food with regard to the fat deposition in the body The Swiss mice on the contrary being of the "lean type have largely the same selection of calories, suggesting that in this strain other mechanisms might be gouverning the body composition

The obese mice become fatter after the period on free selection but with regard to the NMRI and CBA strains the weight was unchanged compared to the previous period on the standard laboratory food The obese Swiss mice however invariably became fatter *with* overweight compared with the preselection period The non obese mice on free selection did not change in weight compared with the non obese animals on the standard food

It is of certain interest to note that even if the selection of food among the different strains varied all animals turned fatter The standard diet as shown in Chapter I promotes growth and has a comparatively high feed efficiency regarding the biological value of the protein In addition this food results in a comparatively low fat content of the body and high protein — fat ratio in the normal mice The protein present in the diets used in the food preference study egg albumin is considered to be a well balanced protein regarding the amino acid composition (Munck 1964, FAO 1965 and Chapter III) The increased content of fat in the carcasses of normal mice on free selection obviously must be caused by the fat or

carbohydrate in the diets In addition one has to bare in mind that the caloric density of the three "synthetical diets was higher than in the standard food The protein was represented by egg albumin in all three diets since it was shown that obese ASw mice without protein would select a diet almost without protein (Larsson 1957)

It could naturally be argued that this type of experiments does not reveal anything but a mixture of gustatory and other phenomenon Against such objections, Richter and Barelare (1938) have concluded "The amazing consistency of the choices made by the animals indicates that the changes do not depend on a trial and error process It would seem far more likely that they are due to an altered chemistry of the taste mechanism which reflects the altered chemistry of the rest of the body during pregnancy and lactation Richter and Barelare (1938) thus suggested that a normal animal would select the best choice of not only common nutrients but also vitamines and minerals In part this was confirmed by Larsson (1957) but still the strain differences found in the present study remains In relation to the results by Richter and Barelare (1938) it is evident that beneficial choices exist during pregnancy and lactation (Larsson 1957) In this case increased nutritional demands naturally exist The present study indicates however that nutritional obesity can be produced with a choice of either high carbohydrate or high fat intakes depending upon the strain used

The significant differences in the selection of diets in normal mice depending on the strain are of interest since it proves that results obtained in one laboratory not necessarily have to be identical with results obtained in other places due to differences in diet composition or the strains used Such differences are likely to exist also in humans Therefore when studying problems concerning nutrition one should avoid cathegorical statements to be true for all individuals without investigating the external environment in relation to the internal especially when studying prophylaxis or treatment of obesity

The differences in body fat contents of normal mice subjected to free selection compared with litter mates on the standard laboratory diet are particularly noticable in the NMRI and CBA mice These two strains in particular on free selection developed what could be called nutritional obesity This is partly also true for the Swiss mice It has to be noted that neither of the strains had a significant increase in body weight compared with the controls on the standard diet In the previous study on free selection it was observed that the obese ASw mice often gained weight for a short period of time to reach a higher weight level (Larsson 1957) During

this period of weight gain, the mice selected a diet extremely high in fat content. A similar observation was noted for the obese Swiss and NMRI mice. These animals selected in the beginning a diet which was high in fat but gradually decreased this intake. The only strain of obese mice that had an appreciable weight increase on the free selection was the Swiss strain. It has been shown previously that the Swiss mice are fairly resistent to nutritional obesity. In these studies the protein quantity and quality have been kept under consideration. On free selection, however, it is obvious that even this strain will develop what could be called nutritional obesity. When the new weight level was reached the obese mice gradually decreased the intake of fat and finally selected a diet with equal caloric participation of carbohydrate and fat (Larsson 1957). In this study as mentioned above this was also found in the present chapter. It was also found that the obese CBA and Swiss mice on free selection continously preferred a diet with high fat content. On the contrary the NMRI mice selected a diet with a comparatively low fat content. Still they become obese.

In the literature comparatively few studies have been concerned with free selection studies in normal and obese animals. Lundback and Stevenson (1947) found a reduced carbohydrate intake after fat feeding in normal rats and in rats with hypothalamic hyperphagia. Mayer (*cf.* 1955) studying the hereditary hyperglycemic obese mice found that these animals developed obesity in diets with high content of carbohydrates. Free selection was not performed in these experiments. Michelsen, Takahashi and Craig (1955) and Barboriak *et al.* (1958) described the development of obesity in rats fed for a long time a high fat diet. Masek and Fabry (1959) could not support these findings when rats were given isocaloric high fat, high carbohydrate or high protein diets. It has to be pointed out however that the animals referred to above were not on free selection but were offered one of the diets and then compared with other animals offered an other food.

In part this explains the discrepancies as being due to the higher caloric density of the high fat diets usually applied. In *ad libitum* fed mice as described by Chapter IV the animals regardless of the strain will increase the body fat content on a high fat diet.

If an animal consuming carbohydrates instead of fat will develop significant increase in body fat content it is obvious that the part of the metabolism going through the carbohydrate lipid metabolic cycle is high. The experiments suggest that this might be the case for the NMRI mice where a comparatively low fat consumption still can produce body fat con

tents of 50 %, calculated on dry weight, even in "normal animals In a study by Fabry *et al* (1964) it was suggested that when the effect of the pattern of food intake on the carcass composition in animals receiving diets with varying fat content was followed one probably has to take into consideration "differences in diet composition and its application, duration of feeding rat strain used *etc* This view is entirely agreed with in the present investigation Further, previous results have revealed that obese ASw mice eat less frequently but in larger meals compared to lean litter mates The present study has also demonstrated strain differences with regard to eating frequencies and to the size of the meals eaten Thus the spontaneous eating frequencies were lower in the CBA and NMRI mice when compared with the Swiss animals In this experiment the standard diet was given to groups of the animals These had in every single animal a lower fat content of the body than the corresponding control

In summary these studies show that the strain differences are powerful in determining the selection of food Still, with a free choice of food all three strains will increase the fat content of the body in a degree which would transfer previously normal mice to the cathegory of obese individuals

GENERAL CONCLUSIONS

The present study was undertaken to study different factors believed to be of importance for the etiology of obesity It ought to be pointed out from the beginning that several factors have proven to be of importance for the physiological regulation of body weight and body composition (cf Larsson 1967)

The strain differences in the mice used in the present investigation suggest the importance of hereditary factors for the etiology of obesity The role of genetic factors for the deposition of fat in adipose tissue has been of importance to those interested in the biology of obesity In mice, particularly two forms of hereditary obesity have been studied, the so called "yellow obesity (Danforth 1927) and the hyperglycemic obesity syndrome (cf Mayer 1955 and Hellman 1965) In the present study some of the interest has been focused towards the genetical importance upon body composition without definite pathological changes *per se* such as abnormalities in blood glucose in the properties of the pancreas and in the adrenal glands (cf Hellman 1965) Thus in the mice used in the present investigation no signs of inborn abnormalities were observed Rather one got the impression that external factors such as different types of food or the exercise varied in effect upon body composition due to the hereditary origin

The relation between body fat and fat free body weight varied in normal individuals due to the strain of mice (Chapter I) This difference could not be due to anything but hereditary differences It was also found that the Swiss mice after 24 h fast lost more weight relatively compared to the two other strains

The quality of the protein ingested seems to be of some importance for determining the body fat content Again the strain characteristics are prominent The CBA mice seem to be more sensitive to the biological value of the protein than the other strains studied regarding the fat deposition of the body Thus the lower the protein quality the higher fat content in the body particularly in the CBA mice This phenomenon is also noted in the other strains but not to the same extent

When the animals are given different foods comparable to the food eaten by the average Swede and in other groups given the suggested

diet by the National Institute of Public Health it is of interest to note that the revision suggested (Blix *et al* 1965) gives very favorable results regarding low body fat content In this connection it is worth mention that a third diet, low in carbohydrate but high in fat, resulted in extremely high content of body fat and low content of body water which of plain physiological reasons could be considered as not desirable

The studies on goldthioglucose obesity reveal that this substance will not behave according to common pharmacological patterns The production of obesity by this substance is very much due to the amount given but the effect of the substance is due to physiological conditions such as the feeding state of the animal On the other hand experiments on alloxan diabetic animals tend to show that the reverse situation is active with regard to the toxicity of the substance

When the mice are exercised the fat content of the body decreases even if the animals are to be regarded as normal because they have not been treated with goldthioglucose In obese animals the exercise has a more pronounced effect, because these mice, regardless of strain always loose weight and body fat

The eating behaviour of the various strains differs significantly Thus the animals with the highest fat content on the standard diet — the CBA mice — have the lowest frequency of feeding which is in line with similar findings in rats The choice of diets plays an important role for the body fat content Thus the mice on free selection of food always have a tendency to be fatter than the corresponding controls

SUMMARY

The present experiments have given evidence for the importance and contribution of the following factors for the etiology of obesity in mice

1) *Genetic factors* play an important role since on a given diet strain differences exist with regard to the deposition of fat and the net release of glycerol and FFA from adipose tissue *in vitro* There is also strain differences in feed utilization and fat deposition when diets with different biological values are studied Significant strain differences in food preferences were also noted Pronounced strain differences were found with regard to the sensitivity to goldthioglucose and the incidence of obesity after administration of this substance

2) *Dietary factors* are also of importance since a food with comparatively low biological value with regard to protein usually leads to low feed utilization and growth At the same time the body fat content has a tendency to rise Generally it was noted that the more fat in a diet the higher is the body fat content when the mice are fed *ad libitum* When the recommendations of the National Institute of Public Health are tried on mice, the body fat content diminishes compared to a diet composed after the average intake of the Swedish people

3) *Muscular exercise*, even when moderately applied has a profound effect on the body fat content which will decrease. The net release of glycerol and FFA from adipose tissue *in vitro* will increase in exercised animals

4) *Goldthioglucose obese mice* regardless of the strain all preferred a diet with high fat content when compared to their lean litter mates

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ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 295

RESERPINE-RESISTANT UPTAKE OF CATECHOLAMINES IN ISOLATED TISSUES OF THE RAT

A histochemical study

By

BERTIL HAMBERGER

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FROM THE DEPARTMENT OF HISTOLOGY KAROLINSKA INSTITUTET
STOCKHOLM SWEDEN

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IVAR HÆGGSTRÖMS TRYCKERI AB
STOCKHOLM 196

To the memory of
Nils Åke Hillarp

CONTENTS

KEY TO THE TABLES

Number of experiments within brackets

In some tables the specific fluorescence intensity in the nerve fibres is denoted by the symbols

+++ = strong ++ = moderate + = weak 0 = none

+++ represents the highest fluorescence intensity obtained in the tissues after incubation with CA which corresponds roughly to the fluorescence intensity in an untreated animal Comparisons should be made only between experiment and corresponding control or between experiments in the same series The difference between *e g* ++ and + is distinctly observable Due to a certain variation in preparations treated identically it was sometimes necessary to bracket the last + The number of + signs is not quantitatively related nor directly related to a given amount of monoamine or L dopa

The fluorescence intensity in the endothelial wall is denoted as + (+) or 0

INTRODUCTION

It is now generally accepted that NA[1] is the transmitter released from sympathetic adrenergic neurons of mammals (see Euler 1956 Iversen 1967) In view of the presence in the brain of NA and DA in neurons with the same characteristics as sympathetic adrenergic neurons it is probable that the CA serve as transmitters in the central nervous system as well (Carlsson Falck and Hillarp 1962 Hillarp Fuxe and Dahlström 1966 a b)

The CA neurons in the peripheral and central nervous systems have synthesizing storing releasing and axon transporting properties in addition to several inactivation mechanisms for the transmitter NA released from adrenergic nerves as well as circulating CA can be inactivated in several ways *i e* by diffusion of transmitter from the site of release by extraneuronally located enzymes such as catechol O methyl transferase and MAO by binding to extraneuronal sites and finally—and perhaps most important—by re uptake into the monoamine neurons (Axelrod 1964 Carlsson 1965 Iversen 1965 b 1967)

The uptake of CA in tissues has been subjected to extensive studies using isotope techniques and biochemical assays In adrenergic nerves two levels of uptake are known to exist One is localized to the level of the cell membrane and is insensitive to reserpine (Hamberger Malmfors Norberg and Sachs 1964 Lindmar and Muscholl 1964 Carlsson and Waldeck 1965 Malmfors 1965 Norberg 1965, Carlsson 1966) A second intraneuronal uptake occurs in amine granules by means of a Mg^{++} ATP dependent and reserpine sensitive uptake storage mechanism (see Carlsson, Hillarp and Waldeck 1963 Stjarne 1964 Carlsson 1965 Dahlström Fuxe and Hillarp 1965 Euler 1966)

In vivo studies on the uptake of CA in the monoamine neurons of the central nervous system are difficult because the amines do

[1] *Abbreviations used* CA = catecholamine(s) NA = noradrenaline DA = dopamine dopa = 3 4-dihydroxyphenylalanine MAO = monoamine oxidase

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Chapter II

METHODOLOGY

A *In vitro* technique

Procedure

ANIMALS Male albino rats (Sprague Dawley), weighing 150—220 g were used The rats were sacrificed under light ether anaesthesia by exsanguination The brain and vas deferens were immediately dissected out and placed in ice chilled medium

MEDIA Generally Krebs Ringer bicarbonate buffer (Umbreit Burris and Stauffer 1951) was used in which the calcium concentration was lowered by 50 % (Axelrod Gordon, Hertting Kopin and Potter 1962)[1] The buffer was saturated with 93.5 % O_2 and 6.5 % CO_2 prior to the experiments and glucose was added to a final concentration of 10 mM Other media used were Krebs Ringer phosphate buffer (Umbreit Burris and Stauffer 1951) and 0.3 M sucrose The ionic composition of the Krebs Ringer bicarbonate buffer was changed in some experiments and all ions were replaced by 0.3 M sucrose in others (see Chap III D) Glucose was sometimes excluded from the medium and in some experiments nitrogen atmosphere was used in the Krebs Ringer bicarbonate (N_2 + CO_2) or phosphate (N_2) buffer (see Chap III, D) To media containing CA or dopa small amounts of ascorbic acid (0.2 mg/ml) and EDTA (0.05 mg/ml) were added

PREPARATION AND INCUBATION OF SLICES After chilling the tissue in the medium for at least a few minutes it was put on a moistened filter paper placed on a hard plastic plate on top of a metal block surrounded by ice Thin slices were prepared with a special carefully cleaned razor blade (Valet 3 blade strips Gillette) and a frosted object slide mainly according to McIlwain

[1] Composition of the Krebs-Ringer bicarbonate buffer in g per liter NaCl 6.923 KCl 0.354 $CaCl_2 \cdot 6H_2O$ 0.[illegible]8 KH_2PO_4 0.16 $MgSO_4 \cdot 7H_2O$ 0.294 $NaHCO_3$ 2.100

and Rodnight (1962) The slices of vas deferens were taken along the organ and were 3—5 mm long Coronal slices of the cerebral cortex sagittal slices of the nucleus caudatus putamen and transverse slices of the hypothalamic area were prepared The slices were placed in the medium for not more than 15 min at 0°C and then moved to 50 ml incubation flasks containing 4 5 ml of medium Generally, two slices from the same tissue were placed in each incubation flask The flasks were gassed and rubber stoppered and then incubated at 37°C under continuous shaking In the experiments with nitrogen atmosphere the flasks were gassed continuously When the uptake after CA or dopa was studied the slices were as a rule first preincubated for 15 min before addition of CA or dopa When the influence of drugs on the accumulation of CA was studied these drugs were always present during preincubation When the effect of exclusion of glucose was studied glucose was also excluded from the preincubation medium Unless otherwise stated, the incubation time was 20 to 30 min after the addition of CA or dopa With longer incubation times the flasks were regassed every hour Controls generally from the same rat as that in the experiments were included The controls were incubated in Krebs Ringer bicarbonate buffer without drugs for the same time as in the experiment and in uptake inhibition experiments uptake controls were also included

Comments

The *in vitro* technique with incubation of tissue slices has earlier been used under a number of experimental conditions (see *e g* Field 1948 McIlwain and Rodnight 1962) In order to preserve the tissues and make especially the brain easier to slice the tissues were immediately immersed in ice-chilled medium The crucial factor is to prepare uniform slices which are thin enough to allow diffusion of substrates and oxygen throughout them In practice the most critical point is to maintain adequate oxygenation The thickness of *e g* a rat cerebral cortex slice should not be more than 0 6 mm (Field 1948) The thickness of the rat cortex outside the white matter is about one mm on the top of the skull (König and Klippel 1963) When two coronary slices were made the second regularly just reached the white matter which implied

that the thickness of the cortical slice was about 0 5 mm or slightly more In other central areas the slices were of about the same thickness Vas deferens slices were easy to cut thinner *i e* about 0 3 mm (approximated from the total thickness of the vas deferens and the number of slices obtained)

The use of the histochemical method provides a possibility of judging the vitality of the tissue, and checking the preparative procedure The accumulation of CA in a nerve terminal suggests that both CA and the substrate for energy generation have reached the terminal since the uptake is energy dependent (*cf* Chap III, D) In slices of the vas deferens cerebral cortex and hypothalamus the distribution of fluorescent nerve fibres after reserpine resistant accumulation of CA was almost invariably homogeneous throughout the section In the nucleus caudatus putamen however a completely different picture was found A fluorescent zone was often seen in the border of the slice whereas the interior showed no specific fluorescence even after high concentrations of CA When evaluating slices from the nucleus caudatus putamen only the outer border was therefore taken into account

The quantitative preservation of NA in the incubation bath at 37 C under the present conditions *i e* with small amounts of ascorbic acid and EDTA was studied (Haggendal and Hamberger 1967) It was shown that under the relevant conditions about 90 % of the added NA was unchanged after one hour

B Histochemical fluorescence method

Procedure

After incubation the slices were placed on small pieces of filter paper and attached by a drop of serum from the animal from which the tissue was taken and immediately frozen in propane chilled with liquid nitrogen The tissue was then freeze dried in a high capacity freeze drier[1] designed by Thieme (1965) The frozen tissue was rapidly moved directly from the propane to the cold plate of the freeze drier and placed in pulverized CO_2 ice with which the cold plate was covered During the first hour CO_2 ice was also placed on top of the freeze drying chambers as a cold trap The freeze drier was generally run for at least 3 h

[1] The freeze-drier is manufactured by HETO Birkerød Denmark

with the lowest obtainable temperature in the cold plate (—40° to —50°C) The temperature was then raised to about —30°C for 6—12 h, whereafter the cold plates were allowed to warm up for at least 2 h before removing the specimens These were treated with formaldehyde gas generated from paraformaldehyde at 80°C for 1 h All tissue specimens from one experiment were always treated with formaldehyde gas in the same reaction vessel For preparation of paraformaldehyde see Chap II C The slices which at this stage were extremely delicate were embedded in paraffin sectioned at 8 μ and mounted in Entellan (Merck) containing a small amount of xylene

For microscopy, a fluorescence microscope (Zeiss Standard Junior) with non fluorescent objectives and a dark field oil condenser was used The light source was a HBO 200 high pressure mercury lamp (Osram) with a 3 or 4 mm Schott BG 12 filter a Zeiss 50 (2 mm) being used as secondary filter Scopix G (Gevaert) was used for microphotography For details of the technique and optical equipment see also Dahlstrom and Fuxe (1964) Norberg and Hamberger (1964) Falck and Owman (1965) and Malmfors (1965)

Comments

The chemistry and specificity of the present histochemical technique have been discussed and established in several papers (Falck 1962 Falck Hillarp Thieme and Torp 1962 Corrodi and Hillarp 1963 1964 Corrodi Hillarp and Jonsson 1964 Corrodi and Jonsson 1965 1966 1967 Caspersson Hillarp and Ritzen 1966 Jonsson 1966 1967 a b c, Ritzen 1966 1967) There is thus little doubt that the specific yellow green fluorescence (with the fluorescence microscopical equipment used) in nerve cell bodies non terminal axons and terminals of peripheral tissues (Norberg and Hamberger 1964 Malmfors 1965 Norberg 1967) and of central tissues (Dahlstrom and Fuxe 1965 Fuxe 1965 a b, Hillarp Fuxe and Dahlstrom 1966 a b) from untreated animals is derived from the 6 7 dihydroxy 3 4 dihydroisoquinolines which are formed from the endogenous CA and formaldehyde gas In the experiments where the endogenous monoamines were depleted with a large dose of reserpine the specific yellow green fluorescence found after incubation with NA DA or dopa was derived analogously

from the corresponding 3,4 dihydroisoquinolines I dopa may have been converted to DA or NA and this will be discussed separately Also DA (or α methyl DA) may have been converted to NA (or α methyl NA) α Methyl NA has been shown to form 6 7 dihydroxy 3 4 dihydroisoquinolines with practically the same fluorescence yield as NA and DA while α methyl DA gives a lower yield (Jonsson 1967 a) The 3 O methylated metabolites of NA or DA do not form strongly fluorescent products with formaldehyde gas (Jonsson 1966, 1967 a) and can be concluded not to interfere markedly under the present conditions Furthermore, the deaminated metabolites do not react with formaldehyde

When evaluating the results of changes in the fluorescence intensity (Chap III) the material to be compared was always freeze dried on the same occasion and treated with formaldehyde gas in the same reaction vessel The intensity and distribution of the specific fluorescence in the experimental material were estimated in the microscope and compared to those of controls The fluorescence intensity and the morphological quality could slightly vary between the separate experimental series in all probability on technical grounds

Model experiments have shown that CA concentration is proportional to fluorescence intensity below a certain concentration and that a further rise in CA concentration gives an unchanged or even slightly diminished intensity (Ritzen 1966) due to quenching of the fluorescence The concentration for the occurrence of quenching in models is about 8000 μg/g wet weight (Ritzen 1966) The average NA concentration in *e g* the peripheral adrenergic terminal has been estimated to be 1000—3000 μg/g wet weight (Dahlstrom Haggendal and Hokfelt 1966) but the local concentration of NA may well be even higher As regards DA terminals of the nucleus caudatus putamen the average concentration of DA *in the varicosities was suggested to be even higher 8000 μg/g* wet weight (Anden Fuxe Hamberger and Hokfelt 1966) Regardless of previous treatment of the tissue it is however difficult to draw any reliable conclusions about the subcellular distribution of the fluorescent products after freeze drying and formaldehyde gas treatment (Ritzen 1967) which makes it difficult to evaluate when quenching occurs in tissue experiments

The general experience is that observed increases and decreases in fluorescence intensity reflect corresponding changes in mono

amine levels (*cf* Corrodi and Jonsson 1967) Such changes in fluorescence intensity are evidently observable after partial depletion of the normal CA stores or accumulation of CA after *e g* reserpine induced depletion of the endogenous monoamines (Malmfors 1965 Norberg 1965 Hillarp Fuxe and Dahlstrom 1966 b Corrodi and Jonsson 1967) Under the present conditions the reproducibility of the method was found to be satisfactory which implies that the fluorescence intensity can safely be regarded as a semiquantitative measure of changes in CA content

C. Standardization of paraformaldehyde[1]

Procedure

The water content of paraformaldehyde (Walker 1964) was determined by the Karl Fischer method (Fischer 1935 Mitchell and Smith 1948 Muroi and Ogawa 1963 Information from Pharmacia Uppsala Sweden) A Karl Fischer electrometric titration apparatus operating by the dead stop method was used (Type E 297, Metrohm AG Herisau Switzerland) 100 to 250 mg of paraformaldehyde (purum Merck) were extracted in at least 15 ml of water free methanol for 10 min and then titrated with a stable Karl Fischer reagent in one solution (Pharmacia Uppsala Sweden) The titre of the reagent was about 4 mg H_2O per ml and was regularly controlled by titration of measured amounts of sodium tartrate (p.a Riedel de Haen $Na_2C_4H_4O_6 \cdot 2H_2O$) At least two determinations were made on each paraformaldehyde

To obtain paraformaldehyde with varying water content it was equilibrated at room temperature in closed vessels containing aqueous solutions of sulphuric acid of varying density (Table I)

Experiments on paraformaldehyde

The sensitivity and reproducibility of the present titrimetric method were tested by measuring the water content of 10 samples from the same paraformaldehyde (Table II)

The paraformaldehyde used for the equilibration experiments

[1] A preliminary report has been given of some of the present experiments (Hamberger Malmfors and Sachs 196[illegible])

TABLE I Relation between relative humidity density and g concentrated sulphuric acid per litre for aqueous solutions of sulphuric acid

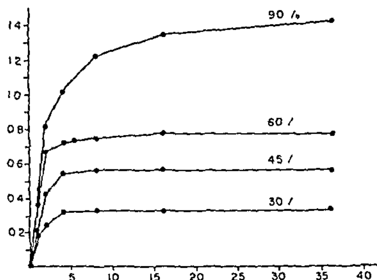
Relative humidity %	Density	Concentrated sulphuric acid g/l
10	1.59	1064
20	1.49	897
30	1.44	779
40	1.39	678
50	1.34	586
60	1.29	503
70	1.25	428
80	1.20	341
90	1.14	231

[1] From Handbook of Chemistry and Physics 30th Edition Chemical Rubber Publishing Co Cleveland 1947 pp 1635 19 6

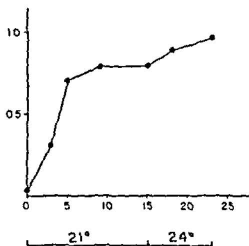
TABLE II Water adsorbed in 10 samples of the same paraformaldehyde equilibrated in 50 per cent relative humidity

Sample	% water
1	0.560
2	0.563
3	0.554
4	0.553
5	0.561
6	0.557
7	0.5[illegible]6
8	0.550
9	0.561
10	0.560

had been dried over phosphorous pentoxide for at least one week Such paraformaldehyde has a water content below 0.05 per cent The *dried paraformaldehyde was equilibrated at room tempera*ture in closed vessels with varying relative humidity obtained from aqueous solutions of varying density (Table I) The result of a typical experiment shows that paraformaldehyde slowly adsorbed water from the atmosphere until it reached a certain level which was dependent on the relative humidity in the closed vessel (Text fig I) As a rule the water content of the paraformal



Text fig I Water content of paraformaldehyde equilibrated at room temperature in closed vessels with different constant relative humidity Dried paraformaldehyde was used The relative humidity was obtained from an aqueous solution of sulphuric acid see table I



Text fig II Water content of paraformaldehyde equilibrated in a closed vessel with 70 % relative humidity obtained from an aqueous solution of sulphuric acid see table I Dried paraformaldehyde was used The equilibration temperature was 21 C for 15 days and was then increased to 24 C

dehyde then stayed constant for a long time although that equilibrated with 90 per cent relative humidity showed a tendency to increase its water content further in some experiments

The equilibration level was also found to be dependent on the temperature during the experiment A typical such experiment is seen in Text fig II After equilibration had been reached at 21°C an increase in temperature to 24°C produced a further increase in water content

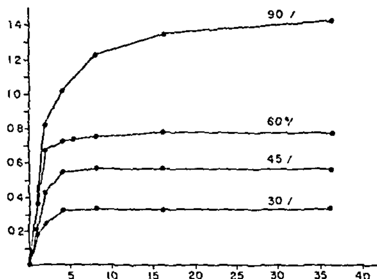
Preparation of paraformaldehyde for routine use

The paraformaldehyde is equilibrated as described above Although it is not as a rule dried before equilibration a constant water content is obtained within a short time and the paraformaldehyde is generally utilized for histochemical experiments after about 10 days In the present *in vitro* experiments the water content ranged from 0 5 to 0 8 per cent The paraformaldehyde was used once only

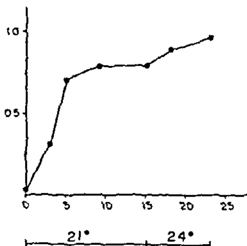
Comments

The sensitivity and reproducibility of the water determination method were found to be satisfactory for the present purpose Although it has been claimed that formaldehyde itself could interfere when applying the conventional Karl Fischer method this is not generally accepted (Mitchell and Smith 1948) Using the Karl Fischer reagent produced by Pharmacia this interference can be disregarded (Information from Pharmacia Uppsala Sweden) Furthermore Muroi and Ogawa (1963) found that with their special Karl Fischer reagent no reaction between methanol and formaldehyde occurred below 30°C

For a satisfactory histochemical result it is necessary for a certain amount of water to be present during the reaction of monoamines with formaldehyde gas while too much water causes diffusion of the monoamines It has not been fully clarified why a certain amount of water must be present during the reaction although the reaction between CA and formaldehyde is a condensation reaction The role played by the water is probably due partly to an increased reactivity of the formaldehyde gas but it might also provide a more optimal pH of the surrounding proteins



Text fig I Water content of paraformaldehyde equilibrated at room temperature in closed vessels with different constant relative humidity Dried paraformaldehyde was used The relative humidity was obtained from an aqueous solution of sulphuric acid see table I



Text f II Water content of paraformaldehyde equilibrated in a closed vessel with 70 relati humidity obtained from an aqueous solution of sulphuric acid see table I Dried paraformaldehyde was used The equilibration temperature was 1 C f [illegible] days and was then increased to [illegible] C

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favouring the existence of the quinoidal form of the 6 7 dihydroxy 3 4 dihydroisoquinolines responsible for the strong fluorescence (Corrodi Jonsson and Malmfors 1966 Ritzen 1967) It is also possible that small amounts of water cause intraneuronal diffusion of the amines (Ritzen 1967)

The water present during the histochemical reaction may derive from the tissue from the air initially enclosed in the reaction vessel and from the paraformaldehyde The influence of tissue water after controlled and efficient freeze-drying and proper handling conditions may be considered constant and probably fairly low Incubation of freeze dried spinal cord in an atmosphere of high relative humidity has been used to increase the tissue water before the formaldehyde reaction (Dahlstrom and Fuxe 1965) but this method is not in general use

Water may also derive from the air initially enclosed in the reaction vessel generally with a volume of 1 litre With 50 per cent relative humidity the air in such a vessel contains about 9 mg of water at 21°C (Handbook of Chemistry and Physics) This water can be controlled and kept on a constant level by using a room with constant relative humidity and temperature

Generally about 6 g of paraformaldehyde are used The amount of adsorbed water available in a paraformaldehyde containing 0 8 per cent water would thus be about 50 mg Judging by the figures of Muroi and Ogawa (1963) about the same amount can be derived from the pyrolysis of paraformaldehyde at 80°C for 1 h Under the histochemical conditions used paraformaldehyde thus seems to be the most important source of water The water originating from the pyrolysis is probably constant since paraformaldehyde is utilized once only Under these conditions the variation in adsorbed water is the crucial factor

For quantitative determinations of catecholamines with the present method it is of great importance to use standardized paraformaldehyde as different humidities give varying fluorescent yields (Norberg Ritzen and Ungerstedt 1966 Ritzen 1966) Use of paraformaldehyde with a high water content may also induce side reactions under certain conditions (Caspersson Hillarp and Ritzen 1966 Ritzen 1966 Jonsson 1967 b)

Paraformaldehyde standardized in the way described has been used in this laboratory for all kinds of tissue since the end of 1963 (see e g Malmfors 1965 Hillarp Fuxe and Dahlstrom 1966

a b Norberg 1967) The histochemical results appear to be dependent above all on the adsorbed water of paraformaldehyde Distinct impairment of the histochemical results was also observed when the climate was hot and humid

The aforegoing data emphasize the necessity of controlling and standardizing all steps in the histochemical technique *i e* freeze drying efficiency handling of tissue before reaction with paraform aldehyde and water content of air and paraformaldehyde Handling of tissue in a room with low and constant relative humidity and constant temperature is highly desirable As in addition a certain variation exists between tissues (*cf* Dahlstrom and Fuxe 1965 Olson 1967) it is advisable to test paraformal dehyde with a water content from 0 3 to 1 2 per cent for practical work

D Drugs

The following drugs were injected intraperitoneally

Reserpine (Serpasil® Ciba) 5—10 mg/kg 12—18 h before killing

Nialamide (Niamid® Pfizer) 100 mg/kg 1—3 h before killing dis olved in 0 9 % NaCl with the help of a small amount of N HCl after which the solution was adjusted to about pH 5 with N NaOH

Unle s otherwise stated the following drugs were dissolved in Krebs Ringer bicarbonate buffer and then diluted in the same buffer The catecholamines and metaraminol were calculated as the base The concentrations used are given

(—) NA (Sigma) 0 01—10 μg/ml

(—) *a* Methyl NA hydrochloride (Corbasil® Hoechst) 0 001—10 μg/ml

DA hydrochloride (Sigma) 0 01—10 μg/ml

a Methyl DA hydrochloride (Hassle) 0 01—10 μg/ml

(—) Metaraminol bitartrate (Aramine®, Merck Sharp & Dohme) 0 1—10 μg/ml

L dopa (Sigma) 0 1—100 μg/ml

D dopa (Sigma) 0 1—100 μg/ml

Reserpine phosphate (Ciba) 10^{-9}—10^{-} M dissolved in 5 % glu cose

Desipramine (Pertofran® Geigy) 10^{-8}—10^{-3}M

(+) Amphetamine sulphate (Sigma), 0.8 $\cdot 10^{-}$—2.7 $\cdot 10^{-}$ M
Chlorpromazine (Hibernal® Leo) 10^{-6}—10^{-3}M
Cocaine hydrochloride 10^{-6}—10^{-3}M
NSD 1015 (3 hydroxybenzyl hydrazine fumarate, Smith and Nephew Research Ltd.) 50 μg/ml
2,4 Dinitrophenol (p.a. Merck) 5 $\cdot 10^{-}$—10^{-3}M
Potassium cyanide (p.a. Merck) 10^{-3}M
Ouabain (g-Strophantin Sandoz) 10^{-3}M

Chapter III

UPTAKE OF CATECHOLAMINES IN VITRO

A Localization of endogenous catecholamines in the relevant tissues of untreated animals

Results

Slices of four model tissues were used for the present experiments vas deferens cerebral cortex hypothalamus and nucleus caudatus putamen The fluorescence microscopical appearance of slices from these areas in untreated animals is described in the following

In slices of the vas deferens a dense network of strongly fluores cent nerve terminals with distinct varicosities was seen within the smooth muscle layers (Figs 1, 3) Bundles of non terminal axons were often present in the outer border of the smooth muscle (Fig 3) The distribution of adrenergic terminals in normal tissues has been thoroughly described earlier (Falck 1962 Norberg and Ham berger 1964 Owman and Sjostrand 1965 Norberg Risley and Ungerstedt 1967)

In slices of various areas of the cerebral cortex very fine fluo rescent terminals (Figs 5, 9) were seen in a network of varying density (*cf* Fuxe Hamberger and Hokfelt to be publ) These terminals are difficult to reproduce regularly unless their fluores cence has been increased by incubation with CA (see below) but have been observed earlier in *in vivo* preparations (Fuxe 1965 b)

Slices of the nucleus caudatus putamen showed a dense network of mainly very fine terminals (*cf* Figs 6 8) The single terminals are often difficult to distinguish in *in vivo* preparations (Fuxe Hokfelt and Nilsson 1965) but can be clearly seen after incuba tion without CA in the medium The single terminals are es pecially easy to distinguish in 1—2 μ thick Araldite sections after incubation with α methyl NA (Andén Fuxe Hamberger and Hok felt 1966)

The microscopical appearance of slices from the hypothalamic area was generally less uniform than that just described A net work of fine to fairly thick terminals of varying density was

always present (Fig 10) The distribution of CA nerve terminals has been thoroughly mapped out (Fuxe 1965 a b)

Incubation of slices from the aforementioned areas in the standard Krebs Ringer medium did not decrease the fluorescence intensity even if incubation was continued for 3 h In a few experiments slices of vas deferens were incubated for 8 h and no decrease in fluorescence was apparent

Incubation of slices from untreated rats with NA up to 1 µg/ml did not markedly change the appearance whereas a larger dose (10 µg/ml) resulted in increased fluorescence (*cf* Angelakos and King 1965) Pretreatment with nialamide (100 mg/kg) before incubation with NA or α methyl NA increased the fluorescence at 1 µg/ml as well After this treatment the bundles of non terminal axons in slices of the vas deferens showed increased fluorescence (*cf* Fig 4) Non terminal axons both in large bundles and singly were also observed in slices from the hypothalamic area (Figs 12 13) In slices of the cerebral cortex (Fig 9) and nucleus caudatus putamen the fluorescent nerve terminals were more distinct after incubation with *e g* α methyl NA

Furthermore incubation of brain slices with CA or L dopa in a concentration of 1 µg/ml or more resulted—provided that the effect of MAO had been excluded—in strong fluorescence in the walls of the capillaries pericytes and/or endothelial cells (Hamberger and Masuoka 1965) The fluorescence in these cells occurred in brain slices of both untreated and reserpine pretreated rats (Figs 18 to 21) and will be described separately in Chap III F

Discussion

The reason for which these experimental tissues were chosen was to obtain representative tissues which are relatively homogeneous with respect to CA content and large enough to provide several slices from each animal It is also highly desirable that the NA and DA nerve terminals can be studied separately The smooth muscle layer of the vas deferens (Falck 1962 Norberg and Hamberger 1964 Owman and Sjostrand 1965 Norberg Risley and Ungerstedt 1967) and the cerebral cortex (see Anden Fuxe and Ungerstedt 1967) contain NA terminals The nucleus caudatus putamen has large numbers of DA nerve terminals (see Anden Fuxe Hamber

ger and Hokfelt 1966) In the hypothalamus the predominant CA is NA which implies that most terminals and non terminal axons normally contain NA (see Fuxe 1965 b) However, DA nerve terminals and non terminal axons are also present in this area e g the tubero infundibular DA neurons (Fuxe 1964 see also Chap III F)

Incubation of slices from untreated animals with *a* methyl NA was used for thorough studies of the appearance and distribution of CA terminals in several areas This is of special value when it is normally difficult to detect the individual terminals, as in the cerebral cortex (Fuxe Hamberger and Hokfelt to be publ), the nucleus caudatus putamen (Anden Fuxe, Hamberger and Hokfelt 1966) and the brown adipose tissue (Wirsen 1965 Wirsen and Hamberger 1967)

B Depletion of endogenous catecholamines

Results

The ability of reserpine and desipramine in varying concentrations to deplete the endogenous CA *in vitro* was investigated The effect of reserpine phosphate on vas deferens slices of untreated rats is seen in Table III A concentration of 10^{-3}M produced a distinct decrease in the fluorescence intensity after 1 h while 10^{-6}—10^{-9}M had a roughly similar effect after 2 h Specific fluorescence was still observed after incubation for 4 *h* with reserpine If the tissue had been exposed to reserpine only for the first 15 min of the total incubation time the decrease was the same as if reserpine had been present throughout incubation 10^{-5}M reserpine phosphate induced a prominent yellow background fluorescence which could be faded by the activation light (Haycock Sheth and Mader 1959)

The effect of desipramine on the vas deferens hypothalamus and nucleus caudatus putamen *in vitro* is shown in Table IV Desipramine in a high concentration (10^{-3}M) had the ability to decrease fluorescence intensity from endogenous CA highly efficiently as no specific fluorescence was found after 15 min 10^{-3}M desipramine and lower concentration did not appear to have any such effect

TABLE III Fluorescence intensity in vas deferens nerve terminals of untreated rats after incubation with reserpine

Reserpine concentration	Incubation time 30 min		60 min		120 min		240 min	
10 [illegible]M	++(+)	(3)[1]	+	(5)[1]	(+)	(5) [2]	(+)	(3)[1, 2]
10^{-4}M	+++	(3)	+(+)	(4)	+	(4)	(+)	(3)
10 [illegible]M	+++	(3)	++	(5)	+	(5)	(+)	(3)
10 [illegible]M	+++	[illegible]	++(+)	(4)	+	(4)[2]	(+)	(3)
10 [illegible]M	+++	[illegible]	++(+)	(5)	+(+)	(5)	+	(3)
0	+++	(8)	+++	(9)	+++	(6)	+++	(3)

For symbols used see key to the tables p 6

Reserpine phosphate was dissolved in 5 % glucose and diluted in Krebs-Ringer bicarbonate medium

[1] Prominent yellow background fluorescence

[2] Same result when exposure time to reserpine was 15 min followed by incubation of the tissue in medium without drugs (3)

TABLE IV Fluorescence intensity in nerve terminals of vas deferens hypothalamus and nucleus caudatus putamen[1] of untreated rats after incubation with desipramine

Desipramine concentration	Incubation time 15 min		30 min		60 min		120—180 min	
10 [illegible]M	0	(3)	0	(3)				
10 [illegible]M			+	[illegible]	0	(3)		
10^{-5}M — 10^{-7}M			+++	(3)	+++	(3)	+++	(3)
0			+++	(6)	+++	(6)	+++	(5)

For symbols used see key to the tables p 6

[1] Two experiments on nucleus caudatus putamen at each point

Discussion

Reserpine is known to prevent the accumulation of CA in amine granules by interfering with the Mg^{++} ATP dependent uptake storage mechanism in the granules and cause the endogenous CA to disappear (see Carlsson Hillarp and Waldeck 1963 Euler and Lishajko 1963 Dahlström Fuxe and Hillarp 1965 Carlsson 1965) The reserpine induced depletion of endogenous NA was slow even slightly slower than in in vivo experiments since detectable amounts were found also after 4 h This could be partly due to

reduction in nervous activity which has been shown to slightly delay the depletory effect of reserpine (Sedvall 1964 Malmfors 1965, Anden Fuxe and Hokfelt 1967)

Exposure of the tissue to reserpine for 15 min only caused the same depletion as if reserpine was present during the whole incubation time This suggests that the interference of reserpine with the uptake storage mechanism has a rapid onset and that the effect cannot be reversed by removing reserpine from the incubation medium (*cf* Dahlstrom and Haggendal 1966)

The depletory effect of desipramine is seen with 10^{-4}M or higher concentrations while with 10^{-5}M no depletion is observed It has also been reported that perfusion of cat hearts with high concentrations of desipramine (above 10^{-4}M) results in a rapid large release of endogenous NA (Titus Matussek Spiegel and Brodie 1966) *In vivo* desipramine in moderate doses does not lower endogenous CA (Carlsson 1966) Recent data have shown that high doses of desipramine and protriptyline can, in fact lower the endogenous CA levels in the brain (Carlsson pers comm) A probable reason for the depletory action of desipramine is blockade of the Mg^{++} ATP dependent uptake storage mechanism in amine granules which at least in adrenal medullary granules occurs in concentrations above 10^{-} M (Carlsson, Hillarp and Waldeck 1963) The very fast effect at 10^{-3}M suggests that at this concentration desipramine directly interferes with the storage of CA

As reported in Chap III E desipramine prevents accumulation of CA in NA but not in DA terminals but its depletory effect seems equally good in both It thus seems probable that the depletory effect of desipramine on endogenous CA differs from the inhibition of uptake in NA terminals

C Uptake of various catecholamines after reserpine pretreatment

Results

All rats used in these experiments were pretreated with reserpine (5 to 10 mg/kg) and killed 12 to 18 h later, and some were

pretreated with nialamide (100 mg/kg) 1—2 h before killing Control tissues were taken from all animal After this treatment no specific fluorescence of CA could be detected The restitution of specific fluorescence in nerve terminals and non terminal axons after incubation of slices with CA or L dopa was investigated

The restitution of specific fluorescence in the vas deferens and cerebral cortex was studied after different incubation times The slices were preincubated for 15 min in the Krebs Ringer bicarbonate buffer after which α methyl NA was added to a final concentration of 1 0 1 or 0 01 μg/ml The incubation time was 5 20 60 or 120 min The restitution in nerve terminals after 20 min is seen in Table V The longer incubation times did not increase the fluorescence intensity appreciably while incubation for 5 min resulted in considerably lower intensity than incubation for 20 min

In the four model tissues both NA and DA restored the specific fluorescence in the nerve terminals provided that the rats had been pretreated with the MAO inhibitor nialamide or their α methylated analogues (Figs 2 4 to 6 8 15) The nerve terminals showed fluorescent varicosities It can be inferred from Table V that the accumulation as reflected in fluorescence intensity of the nerve terminal was in general concentration dependent in the concentration range used This also applied to non terminal axons (Fig 2 4 7 14 15) which after this treatment exhibited fluorescence similar to that of the nerve terminals Strongly fluorescent cell bodies were also occasionally observed (Fig 11) Incubation with 10 μg/ml CA produced general background fluorescence Incubation of slices from the vas deferens or cerebral cortex with NA without pretreatment with a MAO inhibitor resulted in only weak fluorescence in nerve terminal and non terminal axons even at high NA concentrations This fluorescence could be abolished if the tissue was rinsed in the Krebs Ringer bicarbonate buffer without NA for 15 min whereas the fluorescence induced by α methyl NA or by NA after pretreatment with MAO inhibitor did not diminish after such rinsing

In many slices from the hypothalamic area bundles of strongly fluorescent non terminal axons were visible after incubation with CA (Fig 14) In view of their localization (see Chap III A) and the pharmacological results (see Chap III I) part of these non terminal axons in all probability belong to DA neurons As

Table V Fluorescence intensity in nerve terminals after incubation of slices from reserpine pretreated rats with CA

Drugs	Concentration µg/ml	Tissue: Vas deferens		Cerebral cortex		Hypothalamus		Nucleus caudatus putamen	
NA	10	++	(3)	+(+)	(2)			DA 10 ++(+)	(2)
	1	+	(4)	(+)	(3)[1]			DA 1 ++	(2)
	0.1	0	(3)	0	(3)			DA 0.1 (+)	(2)[1]
Nialamide + NA	10	+++	(5)	++(+)	(4)	++(+)	(2)	+++	( )
	1	++(+)	(4)	+	(4)	++	(3)	+++	(4)
	0.1	+(+)	(3)	+	(5)	+	(2)	+(+)	(3)
	0.01	(+)	(3)						
α Methyl NA	10	+++	(6)	++(+)	(4)				
	1	++(+)	(3)	++	(16)	++(+)	(1[illegible])	+++	(14)
	0.1	++	(16)	+	(15)	+(+)	(1[illegible])	+(+)	(13)
	0.03	+	(9)						
	0.01	+	(6)	0	(5)	(+)	(4)	+	([illegible])
	0.003	(+)	([illegible])						
	0.001	0	([illegible])						
Nialamide + DA or α methyl DA	10	++(+)	(5)	++(+)	(5)	++(+)	(4)	+++	(4)[2]
	1	++(+)	(8)	++	(5)	++(+)	(6)	+++	(7)[2]
	0.1	+(+)	(5)	+	(4)	+	(4)	+(+)	(4)
	0.01					0	(2)	+	([illegible])

For symbols used see key to the tables p 6

[1] Incubation for further 15 min without CA abolished the neuronal fluorescence ( )

[2] Incubation for further 15 min without CA had no apparent effect on the fluorescence intensity ([illegible]—3)

previously mentioned (Chap II B) the accumulation of CA was often confined to the peripheral part of slices of the nucleus caudatus putamen (Fig 7)

Incubation of slices of the vas deferens with L dopa after MAO inhibition with nialamide (Table VI) resulted *only in a* weak neuronal fluorescence even after high concentrations of L-dopa which gave a high background fluorescence especially after 100 µg/ml The neuronal fluorescence did not occur in the presence of the dopa decarboxylase inhibitor NSD 1015 Incubation of slices from the cerebral cortex with L dopa in high concentrations (10—100 µg/ml) induced a general yellow green

TABLE VI Fluorescence intensity in nerve terminals after incubation of vas deferens slices with L-dopa. Rats pretreated with reserpine and nialamide were used

L-dopa concentration µg/ml	
100	+ (?)
10	+ (3) [a]
1	(+) (4)
0 1	0 (4)

For symbols used see key to the tables p 6

[a] Increased background fluorescence

[a] Addition of the dopa-decarboxylase inhibitor NSD 1015 50 µg/ml 15 min before L-dopa prevented fluorescence in nerve terminals (3)

background fluorescence Under the present conditions no specific fluorescence was seen in nerve terminals

In all areas of the brain an accumulation of CA in the endothelial walls was observed with a CA or L dopa concentration of 1 µg/ml or above provided that the influence of MAO had been excluded (see Chap III F)

Discussion

There is strong evidence that the CA which accumulates under the present conditions after reserpine induced depletion of the endogenous CA is in fact localized to those nerve fibres that normally contain CA The regional distribution of the fluorescent nerve terminals and non terminal axons after incubation with CA is exactly the same as that reported in untreated animals No new nerve terminal systems were detected although incubation of slices from normal animals with CA often permitted more distinct localization of the terminal Observations on slices from untreated rats also show unequivocally that it is only the nerve fibres with endogenous CA which have the ability to accumulate exogenous CA This is further supported by the finding of the same selectivity in autonomic ganglion cells (Norberg 1965) and of abolishment of the accumulation by sympathetic denervation (Hertting Axelrod Kopin and Whitby 1961 Stromblad and Nickerson 1961 Malmfors and Sachs 1965)

The appearance of the nerve terminals and non terminal axons of both central and peripheral CA neurons in untreated rats and after accumulation is also indistinguishable although non terminal axons and terminals have about the same fluorescence intensity in the latter case In whole mounts of rat iris, the varicosities were less pronounced after depletion with reserpine and accumulation with NA (Malmfors 1965) In sections of slices from the tissues used this is generally difficult to observe and distinct varicose fibres can be seen after reserpine pretreatment as well In *in vitro* experiments on rat iris the same morphological appearance as *in vivo* was however observed (Hamberger and Malmfors 1967) The discrepancies in this respect between the tissues used and the iris are presumably to be ascribed to differences in the structure of these organs In the nucleus caudatus putamen accumulation of CA is mainly confined to the peripheral parts of the slices probably due to the special structural organization of this area

The occurrence of CA uptake after reserpine has earlier been reported both quantitatively (Lindmar and Muscholl 1964, Carlsson and Waldeck 1965 Carlsson 1966 Glowinski Iversen and Axelrod 1966 Iversen 1967) and histochemically (Fuxe and Hillarp 1964 Hamberger Malmfors Norberg and Sachs 1964 Malmfors 1965 Norberg 1965 Fuxe and Ungerstedt 1966) as well as in isolated tissues (Hamberger and Masuoka 1965 Costa Boullin Hammer Vogel and Brodie 1966 Haggendal and Hamberger 1967)

The experiments with varying incubation times showed that the accumulation as revealed by the fluorescence intensity reached a constant level after 20 min under the conditions used Time curves made by use of labelled NA on slices from untreated animals suggest that equilibration is attained later (Dengler Michaelson Spiegel and Titus 1962 Ross and Renyi 1964) This difference may be due to quenching of the fluorescence (see Chap II B) especially after the high concentration and/or differences between untreated and reserpine pretreated animals Furthermore the accumulation of CA in nerve terminals is detected histochemically whereas the total uptake *i e* both neuronal and extraneuronal is measured when using labelled compounds

The experiments with varying concentrations showed a concentration dependent uptake in all tissues and of all drugs The lowest

TABLE VII Effect of different incubation media lowering of temperature and metabolic inhibitors on the accumulation *in vitro* of α methyl NA in nerve terminals of central and peripheral CA neurons in reserpine pretreated rats

All experiments were compared with the accumulation induced by α methyl NA 1 and 0 1 (cerebral cortex hypothalamus nucleus caudatus putamen) or 0 03 (vas deferens) μg/ml in a Krebs Ringer (K R) *bicarbonate medium gassed with* O CO *and containing* glucose (Composition Na 143 mM K 6 0 mM Ca 1 3 mM Mg 1 2 mM see also Chap II A)

Modification of medium and/or addition of drugs	Inhibition in Vas deferens nerve terminals		Central NA and DA nerve terminals	
K R phosphate gassed with O or N	None	(4)		
K R bicarbonate gassed with N CO	None	(3)		
Glucose excluded	None or slight	(10)		
K R bicarbonate gassed with N CO + exclusion of glucose	Marked	(4)	Marked	(2)
0 3 M Sucrose K R medium excluded	Complete	(3)		
Incubation at 0 C in K R bicarbonate	Complete	(4)	Complete	(2)
2 4 Dinitrophenol 5 10^{-4}M + exclusion of glucose	Marked	[illegible]	Marked	[illegible]
2 4-Dinitrophenol 10 M + exclusion of glucose	Marked or complete	[illegible]	Marked	[illegible]
Potassium cyanide 10^{-3}M + exclusion of glucose	Complete	[illegible]		
Ouabain 10 M	Slight or marked	(2)	Marked	[illegible]
K exchanged for Na	Complete	(4)	Complete	(2)
50 % Na exchanged for K	Marked	(3)	Marked	[illegible]
Na exchanged for Li	Complete	[illegible]		
50 % Na exchanged for Li	Marked	[illegible]		
Exclusion of Mg and Ca	None	[illegible]		

Discussion

A number of modifications of the standard experimental set up have been found to interfere with the reserpine resistant accumulation of CA in central and peripheral CA nerve terminals Most experiments were performed on vas deferens slices, and the main points investigated for the central CA terminals The accumulation proved to be dependent on energy yielding processes, since it did not occur without oxygen and glucose and at a low temperature nor after addition of inhibitors of phosphorylating respiration such as dinitrophenol or cyanide The experiments demonstrate that the energy required for CA accumulation can be generated by oxidation of either glucose or endogenous substrates It is also evident that anaerobic glucolysis *per se* can provide sufficient amounts of high energy compounds (*cf* Whittam 1964)

Variations in ionic composition showed that a well balanced 'extracellular ionic requirement was a prerequisite for CA accumulation An absolute requirement appears to exist as no accumulation occurred after incubation with sucrose Marked increases in potassium concentration which produced strong inhibition of accumulation caused depolarization of the cell membrane This suggests that these two mechanisms may be correlated although it is also possible that other mechanisms than depolarization are involved (*cf* Gage and Quastel 1965 Hamberger and Malmfors 1967) On the other hand, cations—except for sodium and potassium—appeared to be of minor importance The optimal sodium and potassium concentrations for CA accumulation resemble those stimulating the sodium potassium activated ATPase of cell membranes (Kurotawa Sakamoto and Kato 1965) Analogously the specific inhibitor of this enzyme ouabain (see *e g* Whittam 1964 Whittam and Blond 1964 Csaky 1965 Quastel 1965), depresses CA uptake

Experiments on the uptake of radioactively labelled noradrenaline (Dengler Michaelson Spiegel and Titus 1962 Iversen and Kravitz 1966 Titus and Dengler 1966) and metaraminol (Giachetti and Shore 1966 Ross and Renyi 1966) in isolated tissues of untreated animals have demonstrated inhibition of uptake under similar conditions The present experiments have shown that the mechanism responsible for this uptake is insensitive to reserpine and is probably localized to the level of the cell membrane (for further discussion see Chap IV)

E Effects of drugs on the uptake of catecholamines

Results

The restitution of specific fluorescence by various catecholamines was described in Chap III, C An account will now be given of the ability of certain drugs to prevent restitution Special interest was focused on a comparison between the NA and DA nerve terminals and non terminal axons

To investigate the possibility that reserpine affects the accumulation of CA after reserpine pretreatment, reserpine phosphate was added to the medium In a concentration of 10^{-6}M it did not affect the restitution *in vitro* of specific fluorescence in the vas deferens induced by α methyl NA (1 and 0 03 μg/ml) 10^{-}[illegible]M reserpine was also used and did not influence restitution although a yellow background fluorescence of reserpine was observed

Table VIII illustrates the ability of desipramine to prevent restitution with α methyl NA Under these conditions desipramine was active down to 10^{-}[illegible] M on NA nerve terminals with α methyl NA (0 03 μg/ml), but the inhibition seemed to be dependent on the concentration of the amine However neither the DA terminals in the nucleus caudatus putamen nor certain bundles of non terminal axons in the hypothalamic area were affected by desipramine (Figs 16 17) not even at a concentration of 10^{-3}M, which depletes endogenous CA (Chap III B) When DA was used after nialamide pretreatment instead of α methyl NA (Table VIII) the same effect of desipramine was noted and the restitution in DA nerve terminal and non terminal axons was uninfluenced

Chlorpromazine also differed in its effect on NA and on DA nerve fibres (Table IX) In concentrations of 10^{-6} and 10^{-}[illegible]M in the incubation medium slight to marked prevention of restitution occurred in NA nerve terminals whereas the DA terminals were unaffected With higher concentrations (10^{-4}—10^{-3}M) however an effect was seen in the DA nerve fibres as well (+) Amphetamine and cocaine prevented restitution with α methyl NA in DA and NA nerve fibres to the same degree (Table X) A marked effect was seen with (+) amphetamine 0.8 10^{-6}M and with cocaine 10^{-}[illegible] M

TABLE VIII Fluorescence intensity in NA and DA nerve terminals and non terminal axons of reserpine pretreated rats after incubation with *a methyl NA in the presence of desipramine*[1]

	α methyl NA							
	1 μg/ml				0 1 or 0 03 μg/ml			
Desipramine concentration	NA nerve terminals and non terminal axons		DA nerve terminals and non terminal axons		NA nerve terminals and non terminal axons		DA nerve terminals and non terminal axons	
10^{-3} M	0	(?)	+++	(2)	0	(2)		
10^{-4} M	0	(6)	+++	(4)	0	(6)		
10 M	(+)	(4)	+++	(4)	0	(6)	++	(?)
10^{-6} M	+	(3)	+++	(1)	0	(3)	++	(-)
10 M	++	(2)			(+)	(3)	++	(2)
10 M	++	(2)			+	(3)		
0	++	(6)	+++	(4)	+	(6)	++	(?)
	Nialamide + DA							
	1 μg/ml				0 1 μg/ml			
10^{-6} M	0	(?)	+++	(?)	0	(-)	++	( )
0	++	(-)	+++	(?)	+	(-)	++	(2)

For symbols used see key to the tables p 6

[1] Results in vas deferens cerebral cortex hypothalamic area and nucleus caudatus putamen

TABLE IX Effect of chlorpromazine on the accumulation of α methyl NA in NA and DA terminals and non terminal axons of reserpine pretreated rats

The effects were compared to the *in vitro* accumulation induced by α metyl NA 1 and 0 1 μg/ml

	Inhibition in			
Chlorpromazine concentration	NA nerve terminals and non terminal axons		DA nerve terminals and non terminal axons	
10^{-5} M	Complete	(?)	Complete	(?)
10 M	Complete	(3)	Slight to marked	(3)
10 M	Marked	(?)	None	(?)
10 M	Slight to marked	(-)	None	(2)

[1] Results in vas deferens cerebral cortex hypothalamic area and nucleus caudatus putamen

TABLE X Effect of (+) amphetamine and cocaine on the accumulation of α methyl NA in NA and DA terminals and non terminal axons of *reserpine pretreated rats*

The effects were compared to the *in vitro* accumulation induced by α methyl NA 1 and 0 1 μg/ml

(+) Amphetamine concentration	Inhibition in NA and DA nerve terminals and non terminal axons	
2.7 10 M — 2.7 10^{-4}M	Complete	(3)
0.8 10^{-4}M	Marked	(3)
2.7 10 M	None or slight	(3)
0.8 10 M	None	(-)
Cocaine concentration		
10^{-4}M	Complete	(-)
10 M	Marked	(3)
10 M	Slight	(-)
10^{-4}M	None or slight	(?)

[1] Results in vas deferens cerebral cortex hypothalamic area and nucleus caudatus putamen
[2] This concentration had an effect only on the terminals in the cerebral cortex

Discussion

The drugs used in the present experiments are known to inhibit the accumulation of CA both in the peripheral (see *e g* Hertting Axelrod and Whitby 1961 Axelrod 1964 Carlsson and Waldeck 1965 Malmfors 1965 Iversen 1967) and in the central nervous system (see *e g* Dengler Spiegel and Titus 1961 Carlsson 1966 Carlsson Fuxe Hamberger and Lindqvist 1966 a Glowinski Axelrod and Iversen 1966) The present experiments have shown that the *in vitro* uptake into the CA nerve terminals and non terminal axons is inhibited Furthermore the data demonstrate differences between the accumulation in NA and DA neurons Thus desipramine prevents accumulation in NA nerve terminals and non terminal axons only while those normally containing DA are unaffected even at a high concentration (10^{-3}M) which depletes endogenous DA (Chap III B) (see also Carlsson Fuxe Hamberger and Lindqvist 1966 a Fuxe Hamberger and Malmfors 1966 Hamberger 1966 Häggendal and Hamberger 1967) The selective effect of desipramine provides a new possibil

ity of differentiating between NA and DA neurons in the central nervous system (Fig 17) The same inhibition pattern as that found with desipramine applies to other imipramine like drugs such as protriptyline (Carlsson Fuxe, Hamberger and Lindqvist 1966 a) It is also evident from these results that the inhibitory effect of desipramine is dependent on the concentration of α methyl NA Thus a 10 to 30 fold increase in NA concentration requires a 100 fold increase in desipramine concentration to achieve the same inhibitory effect i e no accumulation can be detected (Table VIII) Studies with radioactive NA on uptake inhibition in cat heart slices showed an effect of desipramine down to 10^{-12}M (Titus Matussek Spiegel and Brodie 1966)

(+) Amphetamine and cocaine prevent uptake in both NA and DA nerve fibres while chlorpromazine affects both NA and DA nerve terminals although the former at a lower concentration For further discussion see Chap IV

F Accumulation of catecholamines in the endothelial wall

Results

Incubation of brain slices with CA (1 μg/ml) resulted in intense specific fluorescence in the endothelial wall provided that the effect of MAO had been prevented (Table XI) The fluorescence was localized to cells in the endothelial wall (Figs 18 to 21) mainly pericytes although specific fluorescence was also visible in the endothelial cells especially after 10 μg/ml (see Barka and Anderson 1962 Landers Chason Gonzalez and Palutke 1962 Wolff 1963 Bertler Falck Owman and Rosengren 1966 Schwink and Wetzstein 1966) This type of fluorescent cell in the endothelial wall was not observed outside the central nervous system In concentrations of 1 and 10 μg/ml both DA and NA and their α methylated analogues induced fluorescence while 0 1 μg/ml produced none Metaraminol (1 to 10 μg/ml) produced yellowish fluorescence in the cells in question

Incubation with L-dopa also induced strong fluorescence in the endothelial wall (Table XII) The fluorescence did not occur if the tissue had been preincubated with the dopa decarboxylase inhibitor NSD 1015 (50 μg/ml) Incubation with D dopa also induced specific fluorescence although weaker than that of L dopa

TABLE XI Fluorescence intensity in the endothelial walls of cerebral capillaries after incubation of cerebral cortex slices with CA or metaraminol

Drug	Concentration $\mu g/ml$		
NA	10	0	(2)
	1	0	(3)
α Methyl NA or nialamide + NA	10	+	(8)
	1	+	([illegible]0)
	0 1	0	(20)
α Methyl DA or nialamide + DA	10	+	([illegible])
	1	+	(15)
	0 1	0	(4)
Metaraminol	10	+	(3)
	1	(+)	(3)
	0 1	0	(3)

For symbols used see key to the tables p 6

[1] Rats pretreated with reserpine

Incubation for further 15 min without CA had no apparent effect on the fluorescence intensity (2)

TABLE XII Fluorescence intensity in the endothelial walls of cerebral capillaries after incubation of cerebral cortex slices[1] with dopa

L-dopa concentration $\mu g/ml$			D-dopa concentration $\mu g/ml$		
100	+	([illegible])	100	+	(2)
10	+	([illegible])	10	0	(2)
1	+	(4)	1	0	([illegible])
0 1	0	([illegible])			

For symbols used see key to the tables p 6

Rats pretreated with reserpine and nialamide

Addition of the dopa-decarboxylase inhibitor NSD 1015 50 $\mu g/ml$ 15 min before L-dopa prevented fluorescence in the endothelial walls (3)

The effect of modification of media and addition of drugs was also investigated Exclusion of oxygen and glucose or incubation at a low temperature or addition of dinitrophenol in combination with exclusion of glucose all affected the occurrence of fluorescence

TABLE XIII Fluorescence intensity in the endothelial walls of cerebral capillaries after incubation of cerebral cortex slices[1] with α methyl NA in the presence of drugs
α Methyl NA 1 μg/ml was used in all experiments

Drug	Concentration		
Desipramine	10^{-7} — 10^{-4} M	0	(4)
	10^{-8} — 10^{-6} M	(+)	(7)
	10 — 10 M	+	(3)
(+) Amphetamine	2.7 10^{-6} M	0	(2)
	0.8 10^{-6} M	(+)	( )
	2.7 10^{-7} M	+	(2)
Cocaine	10 — 10^{-4} M	0	(5)
	10 — 10^{-6} M	+	(3)
No drug		+	(15)

For symbols used see key to the tables p 6
[1] Rats pretreated with reserpine

similarly to the effects in central nerve terminals (see Table VII) Desipramine (+) amphetamine and cocaine prevented the occurrence of specific fluorescence in the endothelial wall (Table XIII)

Discussion

Cells in the endothelial wall of capillaries in the central nervous system accumulated CA *in vitro* when incubated in concentrations of 1 μg/ml or higher provided that the influence of MAO had been eliminated (see also Hamberger and Masuoka 1965) No differences were found between untreated (Chap III A) and reserpine pretreated rats The mechanism underlying this accumulation in central capillaries is presumably specific since inhibition of energy yielding processes prevented accumulation The concentration ability is however far below that of the nerve terminals and non terminal axons Cells in the endothelial wall were also observed to accumulate metaraminol since it can be visualized extraneuronally due to fluorescence with an emission peak at 510 mμ (Sachs 1965 Jonsson and Ritzen 1966)

Fluorescence after incubation with L dopa was found with about

the same concentrations as those of CA As the dopa decarboxyl ase inhibitor NSD 1015 prevented fluorescence it is likely that the fluorescence is due to a CA probably DA, and that L dopa itself cannot accumulate The same observations have earlier been made *in vivo* (Bertler, Falck and Rosengren 1963 Bertler, Falck Owman and Rosengren 1966) The fact that D dopa induces less intense fluorescence than L dopa may be due to the specificity of the dopa decarboxylase

It is interesting to note that drugs which block the reserpine resistant accumulation in nerve terminals such as desipramine cocaine and (+) amphetamine are also able to block the extra neuronal accumulation The drug concentration for blocking these two types of accumulation is about the same which must be borne in mind when evaluating biochemical measurements on *e g* accumulation of CA and its inhibition(*cf* Häggendal and Hamberger 1967)

It is tempting to assume that the cells in the endothelial wall play a role in the transfer of CA between blood and brain and thus take part in the blood brain barrier for CA No fluorescence is found even after very high *in vivo* doses of CA (Bertler Falck and Rosengren 1963 Bertler Falck Owman and Rosengren 1966) After mild chemically induced blood brain barrier damage howev er CA administered *in vivo* can accumulate in these cells (Ham berger and Hamberger 1966 Flodmark Hamberger Hamberger and Steinwall to be publ)

GENERAL DISCUSSION

In most studies, the uptake of CA has been detected by measuring the accumulation in whole organs or parts of them As pointed out by Iversen (1965 b) the distinction between uptake and accumulation is important in connexion with CA Uptake refers to the passage of a substance from extra to intracellular space but is not primarily related to its storage, which also means that accumulation must include uptake Injection of labelled NA into an untreated animal results in an uptake and accumulation of the injected amine (see *e g* Axelrod 1964 Iversen 1965 b 1967) After reserpine pretreatment no accumulation of NA can be detected although the uptake is unaffected (Lindmar and Muscholl 1964 Iversen Glowinski and Axelrod 1965) Administration of an MAO inhibitor however permits detection of an accumulation of the CA (Fuxe and Hillarp 1964 Hamberger Malmfors Norberg and Sachs 1964 Carlsson 1965 Carlsson and Waldeck 1965 Malmfors 1965 Norberg 1965 Carlsson 1966) There is now strong evidence that reserpine neither *in vivo* after different times (Carlsson 1965 Dahlström Fuxe and Hillarp 1965 Malmfors 1965) nor *in vitro* affects this uptake

After incubation of vas deferens slices from reserpine pretreated rats with *α* methyl NA (0 003—0 01 μg/ml) weak specific fluorescence occurs in the nerve terminals From model experiments (Ritzen 1966) the average intraneuronal NA content must be assumed to be about 10 μg/g wet weight in this case as the relative fluorescence yield of *α* methyl NA and NA is the same (Jonsson 1967 a) When comparing the concentration of CA outside the nerve terminal and the average concentration inside a difference of at least 1000 times is found These calculations are naturally unsafe but receive support from earlier calculations (Hamberger and Masuoka 1965)

The reserpine resistant uptake was characterized in Chap III D The process was found to be energy dependent since the addition of dinitrophenol or cyanide inhibited the accumulation

Incubation at 0°C or in the absence of oxygen and glucose markedly suppressed the uptake The membrane ATPase inhibitor ouabain also prevented accumulation when used in a high concentration The experiments on the ionic composition of the medium showed that a certain concentration of sodium and potassium ions was necessary for uptake of CA As pointed out in Chap III C, experiments on prevention of uptake of NA (Dengler Michaelson Spiegel and Titus 1962 Iversen and Kravitz 1966 Titus and Dengler 1966) or metaraminol (Giachetti and Shore 1966 Ross and Renyi 1966) have previously been made with *e g* metabolic inhibitors and ouabain and the results are supported by the present ones In those studies it is apparent that the initial uptake was dependent on the reserpine resistant mechanism even if the uptake and storage in amine storage granules were intact (*cf* Lindmar and Muscholl 1964) which implies that the same mechanism was studied as in the present study When taking all these facts into consideration there is thus little doubt *that the reserpine resistant uptake of CA fulfills the demands of* an active carrier mediated transport (see Wilbrandt and Rosenberg 1961 Schanker 1962 Csaky 1965)

The reserpine resistant uptake mechanism of catecholamines has been shown to exist in the whole CA neuron *i e* cell body non terminal axon and terminal (Fuxe and Hillarp 1964 Hamberger Malmfors Norberg and Sachs 1964 Malmfors 1965 Norberg 1965 Fuxe and Ungerstedt 1966) despite the varying content of amine storage granules (see the following) This favours the view that it is localized to the level of the cell membrane This view is also borne out by pharmacological experiments Desipramine is known to inhibit the reserpine resistant uptake in NA neurons (see Chap III D) but does not affect the uptake into amine storage granules except in much higher concentrations (Carlsson Hillarp and Waldeck 1963) In animals depleted of their endogenous CA with an inhibitor of the CA synthesis which does not interfere with the Mg^{++} ATP dependent storage in the amine granules desipramine prevents exogenous CA from entering the NA nerve terminals and reaching the uptake storage sites (Malmfors pers comm)

That the amine storage granules are the main site of the reserpine resistant uptake is further contradicted by the finding that after reserpine resistant accumulation of NA non terminal axons

and terminals have a fluorescence of similar intensity despite a low and high content of amine storage granules respectively (Richardson 1964 Hokfelt 1967) These findings make it highly probable that most of the CA accumulated after reserpine pretreatment is localized outside the amine storage granules and more or less free in the cytoplasm It nevertheless seems possible that the CA which accumulates after reserpine pretreatment is partly bound to an intraneuronal structure It has been shown that isolated amine storage granules can take up CA also after reserpine treatment provided that the CA concentration in the incubation medium is high (Stjarne 1964 Euler and Lishajko 1965) It is highly probable that a high intraneuronal concentration can occur under the present experimental conditions In studies of the subcellular distribution of NA taken up after reserpine a certain amount of NA was found in the amine storage granule fraction (Lundborg and Stitzel 1967, Stitzel and Lundborg 1967) Histochemical experiments have suggested that part of the exogenous CA after reserpine is localized to intraneuronal structures with the same distribution as those normally containing NA *i e* the amine storage granules (Hamberger and Malmfors 1967) The mechanism responsible for this supposed binding must however be independent of the Mg^{++} ATP dependent mechanism in amine storage granules

The high concentrations of CA (10—1 μg/ml) used in the present study are considerably higher than those used in studies with radioactive NA (Dengler Michaelson Spiegel and Titus 1962 Titus Matussek Spiegel and Brodie 1966) When using these high concentrations the authors found the same CA concentration in both slice and medium It is however evident from the present results that a large concentration difference certainly exists inside and outside the nerve terminals even with high CA concentrations in the incubation medium The failure of earlier studies to reveal an uptake of CA at high concentrations may be due to the greater amount of CA being present extraneuronally, and masking the specific uptake (*cf* Haggendal and Hamberger 1967) As the CA present intraneuronally more easily diffuses out of the slice if the amines are removed from the incubation medium careful rinsing of the slices after incubation with radioactive CA would in all probability

di close accumulation of isotope even with high CA concentrations in the medium

The CA concentrations used in the present experiments are probably comparable to those normally present just outside the cell membrane of a varicosity when transmitter is released by nerve impulses It may be justified to make some calculations of this matter One varicosity in the rat iris or vas deferens contains about 5 10^{-3} pg NA (Dahlstrom Haggendal and Hokfelt 1966) Depletion of endogenous CA can be achieved by nerve impulses and about 30 000 impulses are needed for this purpo e provided that CA synthesis has been inhibited (Malmfors pers comm) Kernell and Sedvall (1964) reported that in the cat 50 000 impulses are needed to give a 55 to 70 per cent reduction in the endogenous NA with synthesis and re uptake intact Assuming from these data that 10 000 to 50 000 impulses are needed to deplete endogenous NA if no re uptake or synthesis occurs that the varicosity is a sphere about 1—1 5 μ in diameter and that the transmitter released is evenly distributed in a 200—300 Å thick layer (*cf* Taxi 1965) around the whole varicosity one nerve impulse should liberate about 1—5 10^{-7} pg NA and when no uptake occurs the concentration of CA just outside the varicosity should be of the order of 0 5—8 μg/ml This suggests that the concentrations used in the present study may well be compatible with those occurring locally under physiological conditions

In experiments on isolated perfused rat hearts Iversen (1963 a b) found a new catecholamine uptake process (Uptake$_2$) which was detectable with high NA concentrations (1—40 μg/ml) in the perfusion medium The properties of Uptake$_2$ differed from those of Uptake$_1$ which operated at low perfusion concentrations of NA (below 1 μg/ml) and showed similarities to the uptake discussed in the present paper It was suggested that both Uptake$_1$ and Uptake$_2$ were localized to the adrenergic nerves (Iversen 1965 b) However histochemical experiments recently carried out in our laboratory have provided strong evidence that most of the CA taken up by Uptake$_2$ is localized extraneuronally to cells in the connective tissue and probably to the muscle cells (Malmfors pers comm) The suggestion that Uptake$_2$ is related the uptake in the non terminal axons and cell bodies (Iv 1965 b) is strongly contradicted by the finding that the wh

adrenergic neuron reacts uniformly on treatment with drugs (Malmfors 1965 Norberg 1965)

The inhibition of uptake by drugs has shown that, in all probability differences exist in the reserpine resistant accumulation of CA in NA and DA neurons While desipramine inhibited uptake in NA neurons, no effect at all could be found in DA neurons not even in very high concentrations Chlorpromazine inhibited uptake in both NA and DA nerve terminals although higher concentrations were needed for an effect on the latter Cocaine and (+) amphetamine on the other hand, prevented uptake in NA and DA nerve terminals and non terminal axons to the same extent (cf Haggendal and Hamberger 1967) In connexion with (+) amphetamine a releasing activity is probably involved in the prevention of accumulation of CA (Carlsson and Waldeck 1966 Carlsson Fuxe Hamberger and Lindqvist 1966a Hamberger and Malmfors 1967) At least with high (+) amphetamine concentrations, an inhibition of uptake is likely to have occurred since no intraneuronal amine could be detected although the tissue was frozen directly after incubation with CA The same inhibition pattern in DA nerve terminals has been obtained *in vivo* (Fuxe Hamberger and Malmfors 1966) and by use of intraventricular injections (Fuxe and Ungerstedt pers comm) Intraventricular injections of labelled NA gave almost similar results although they were not interpreted in this way (Glowinski Axelrod and Iversen 1966) On the basis of the present pharmacological data it is thus highly probable that the mechanisms for reserpine resistant accumulation in DA and NA nerve fibres differ The nature of this difference remains to be solved The present experiments have also provided strong evidence that NA nerve fibres in the central and peripheral nervous system behave similarly in isolated tissues with respect to uptake and accumulation of CA and its prevention by drugs

The *in vitro* experiments performed thus show that central CA nerve terminals have a highly efficient mechanism for accumulating CA as earlier known for the sympathetic adrenergic neuron This mechanism is presumably located to the level of the cell membrane is insensitive to reserpine, and fulfills the demands of a carrier mediated transport It seems probable that this mechanism constitutes one of the most important modes for inactivation of the released transmitter (Axelrod 1964 Hatch Hürlimann

and Thoenen 1964 Iversen 1967) Furthermore this mechanism appears to be of great importance in psychopharmacology, as certain drugs known to inhibit the uptake of CA are effective antidepressants (Carlsson, Fuxe Hamberger and Lindqvist 1966 a see Garattini and Dukes 1967) This implies that the present technique can be used in a search for new antidepressant drugs

SUMMARY

The uptake and accumulation of CA have been studied in isolated tissues mainly from reserpine pretreated rats After incubation of thin slices of the brain and vas deferens with dopa or CA in a Krebs Ringer bicarbonate buffer the slices were investigated by the histochemical fluorescence method of Falck and Hillarp This method permits the visualization of dopa and CA as NA and DA In the present work the basis for standardization of the form aldehyde gas treatment which is a crucial step in the technique is also described

The experiments performed *in vitro* show that NA and DA terminals and non terminal axons in the brain have a reserpine resistant mechanism for accumulating CA as earlier known to apply to the peripheral adrenergic neuron This mechanism which is probably localized to the level of the cell membrane is shown to have a high concentration ability, to be dependent on energy yielding processes and to require a certain extra cellular concentration of sodium and potassium It is postulated that the reserpine resistant accumulation of CA is an active carrier mediated transport

The inhibition of CA accumulation by drugs was studied with special interest focused on a comparison between NA and DA neurons Whereas (+) amphetamine and cocaine prevented accumulation of CA in both NA and DA terminals and non terminal axons to about the same extent desipramine inhibited uptake only in those normally containing NA Desipramine is found to have no effect on DA terminals and non terminal axons not even in very high concentrations which deplete endogenous NA or DA within 15 min

Apart from the described uptake and accumulation in nervous elements it is found that cells in the endothelial wall pericytes and possibly also endothelial cells in the brain capillaries accumulate CA after incubation with dopa or CA provided that MAO is inhibited This accumulation was energy dependent and could be prevented by drugs, such as desipramine (+) amphetamine and cocaine and it is suggested that these cells play a role in the blood brain barrier for CA

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FIGURES



Fig. 1 Slice of vas deferens, untreated rat, incubated in standard medium without drugs for 1 h. Nerve terminals with strongly fluorescent varicosities. × 100



Fig. 2 Slice of vas deferens, reserpine-pretreated rat, incubated with α-methyl NA (1 μg/ml). Nerve terminals as well as bundles of non-terminal axons (→) both exhibiting strong specific fluorescence. × 100



Fig. 3 Slice of vas deferens untreated rat incubated in standard medium without drugs for 2 h. Above the strongly fluorescent nerve terminals a bundle (→) of non terminal axons with weak fluorescence × 160



Fig. 4 Slice of vas deferens reserpine pretreated rat incubated with α methyl NA (0.1 μg/ml). Nerve terminals with fluorescent varicosities and on the right both single and bundles of non terminal axons × 210

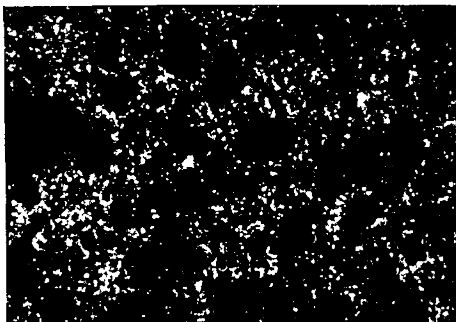


Fig 6 Slice of nucleus caudatus putamen reserpine-pretreated rat incubated with α methyl DA (0 1 μg/ml) High density of strongly fluorescent varicosities of DA nerve terminals The dark areas are non fluorescent nerve cell bodies × 400



Fig 7 Slice of nucleus caudatus putamen reserpine pretreated rat incubated with α methyl NA (1 μg/ml) On the left DA nerve terminals which have accumulated the amine to a varying degree so that the peripheral part of the slice (A) has higher fluorescent intensity Single fluorescent non terminal axons in the tissue



Fig 8 Slice of *nucleus caudatus putamen* reserpine pretreated rat incubated with α methyl NA (1 μg/ml) Extremely dense network of nerve terminals with distinct varicosities The large dark areas are trunks of non terminal axons and the small ones nerve cell bodies × [illegible]



Fig 9 Slice of cerebral cortex untreated rat incubated with α methyl NA (1 μg/ml) Several strongly fluorescent nerve terminals with distinct varicosities × 230



Fig 10 Slice of hypothalamic area untreated rat incubated in the standard medium without drugs for 30 min A network of fine to fairly thick terminals [illegible]



Fig 11 Slice of hypothalamic area reserpine pretreated rat incubated with α methyl NA (1 μg/ml) Several nerve cell bodies with strong fluorescence × [illegible]10

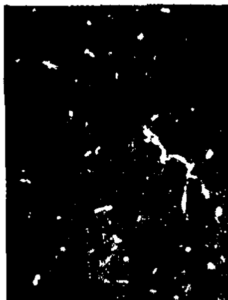


Fig 18 Slice of hypothalamic area un treated rat incubated with α methyl NA (3 μg/ml) Fluorescent cells in the en dothelial wall are confined to the capil laries whereas the larger vessel has no such cells × 210



Fig 19 Slice of nucleus caudatus puta men reserpine and nialamide pretreated rat incubated with DA (1 μg/ml) Fluo rescent cells in the endothelial wall At (→) a typical pericyte bulging out × 330



Fig 20 Slice of hypothalamic area rese pine-pretreated rat incubated with α methyl NA (1 μg/ml) Specific flu rescence in the endothelial wall At (→) a typical pericyte × 210



Fig 21 Slice of cerebral cortex untreated rat incubated with α methyl NA (10 μg/ml) After freeze-drying and formal dehyde gas treatment embedded in Araldite® and sectioned at 1 μ (These specimens were kindly prepared by Dr

ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 296

THE RELATION BETWEEN THE ELECTRICAL AND MECHANICAL ACTIVITY OF HUMAN INTERCOSTAL MUSCLES DURING VOLUNTARY INSPIRATION

HELSINKI 1967

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Supplementum 296

FROM THE INSTITUTE OF PHYSIOLOGY
UNIVERSITY OF HELSINKI
HELSINKI FINLAND

THE RELATION BETWEEN THE ELECTRICAL AND MECHANICAL ACTIVITY OF HUMAN INTERCOSTAL MUSCLES DURING VOLUNTARY INSPIRATION

BY
ANTTI A VILJANEN

HELSINKI 1967

PREFACE

This investigation was carried out at the Institute of Physiology University of Helsinki The problem was suggested by the Head of the Institute, Professor P M Bergstrom M D who generously placed the equipment of the institute at my disposal I wish to express my deep gratitude for his constant advice and encouragement as well as for the critical comments he made during the course of the work

I am also greatly indebted to Docent Hakan Poppius M D for his valuable advice in regard to both the theoretical and technical aspects of the study

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My sincere thanks are due to all members of the staff of the Institute of Physiology and especially to Mr Roger W F Blafield C E for his skillful technical assistance and Mr Pekka Halttunen B M for his continuous interest and valuable assistance during time consuming experiments I am also grateful to Mr Martti Halumaki M D Mr Kyro-Niemi M D Mr Seppo Pyrhonen B M and Mr Lauri Laitinen

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My wife is entitled to my warmest gratitude, her untiring support and understanding made the successful conclusion of the work possible.

Helsinki, April 1967

ANTTI A. VILJANEN

CONTENTS

INTRODUCTION

In studies of the regulation of human respiration one general opinion often expressed is that the movements involved express the intrinsic activity of the respiratory centres. However knowledge is necessary of the relations existent between the neuronal activity and the mechanical activity of the respiratory muscles. It has earlier been demonstrated that in animals the electrical activity of the nervus phrenicus bears a linear relation to the activity of the diaphragm recorded electromyographically (DITTLER 1910, DITTLER & GARTEN 1912, GASSER & NEWCOMER 1921). If knowledge is to be gained of the activity of human respiratory centres it is first necessary to know the laws which bind the electrical activity of the respiratory muscles and the corresponding mechanical activity.

In this study an attempt was made to solve the problem posed by the relations between the electrical and mechanical activity of human intercostal muscles during voluntary inspiration. Experiments were also conducted with CO_2 stimulation. The electrical activity was registered during inspiration by means of a simple electromyogram of the external intercostal muscles. Since the mechanical activity of the external intercostal muscles cannot be measured directly in man the total mechanical activity of the inspiratory muscles was calculated instead.

It may be that knowledge of the relationships which exist between the electrical and mechanical activities determined in this way will lead to further progress in studies which aim at solution of the problems connected with the neuronal regulation of respiration. Moreover the information so gained may be applicable in the monitoring of respiratory functions.

SURVEY OF LITERATURE

It has been shown that an integrated electromyogram registered with surface electrodes bears a linear relationship to the tension produced by a voluntary isometric contraction in human muscle (BAYER & FLECHTENMACHER 1950, INMAN et al. 1952, LIPPOLD 1952). Furthermore it has been reported that integrated action potentials recorded by surface electrodes from the human gastrocnemius muscle during voluntary contractions are directly proportional to the tension when the velocity of shortening or of lengthening remains constant. At a constant tension the electrical activity increases linearly with the velocity of shortening (BIGLAND & LIPPOLD 1954 a). A linear relationship has also been demonstrated between the discharge frequency of the electromyogram recorded from the forefinger abductor muscle, applying a non-selective electrode technique, and the isometric tension produced by abduction (JALAVISTO et al. 1958). JALAVISTO et al. have also shown that the impulse frequency increases with velocity during isotonic shortening.

BERGSTRÖM in his studies of the human forefinger abductor muscle by a non-selective surface electrode method has later shown that 1) a linear relationship exists between the number of motor impulses and the integrated electromyogram activity (1959 a), 2) the number of motor impulses in nearly isometric contractions (»leichte Aktionen») bears a linear relationships to the mechanical force impulse (1958), 3) the discharge frequency of the electromyogram is directly proportional to the kinetic energy produced by the abduction, with the kinetic energy calculated from a movement of a pendulum after a touch of the forefinger and the electromyogram frequency recorded during 0.01 second immediately prior to the moment of detachment (1959 b), 4) the impulse frequency of the electromyogram is directly proportional to the degree of muscle shortening at different loadings of the contractions, and the amount of work produced by unloaded contraction, and furthermore to the estimated work the muscle must perform for attainment of the

abduction degree (1959 c) and 5) the number of electromyogram impulses is directly proportional to the physical action (energy × time dimension g × cm × s^{-1}) of the mechanical effect of the motion (1959 b). Subsequently in electromyogram recordings from the human musculus soleus with two copper wire electrodes it has been shown that 1) the integrated electrical activity bears a linear relationship to the number of electromyogram impulses recorded by an electrical counter, 2) in isometric contraction this tension is directly related to the action potential count or frequency 3) in isotonic contractions there is »an almost linear relationship between total action potential count and work» 4) and if this distance and the duration of contraction are constant, the action potential count is proportional to the average tension (CLOSE NICKEL & TODD 1960).

In evaluation of the results cited it is important to note that even if a linear relationship is found between the tension and the electrical activity of human skeletal muscle by the application of a non selective technique when in fact the activity of several motor units is being recorded the situation changes when the technique is sufficiently selective to give the activity of a single motor unit (JALAVISTO et al 1938 BIGLAND & LIPPOLD 1954 b) BIGLAND & LIPPOLD have further indicated that the frequency of one unit is related to the strength of contraction giving an S shaped curve

A number of research workers (ADRIAN & BRONK 1929 SMITH 1934 LINDSLEY 1935 GILSON & MILLS 1941 BIGLAND & LIPPOLD 1954 b) have reported on the absence of any electrical activity in the relaxed muscle

The gradation of the voluntary contraction of skeletal muscle in man has been shown to originate in changes in the discharge frequency, the duration of motor discharge and the number of active motoneurons (ADRIAN & BRONK 1929 SMITH 1934 LINDSLEY 1935, GILSON & MILLS 1941 TOKIZANE KAWATA & TOKIZANE 1952 BIGLAND & LIPPOLD 1954 b) This has further been demonstrated in gradation of the contraction of the striated skeletal muscle in animals (ADRIAN & BRONK 1929 BRONK & FERGUSON 1935 ATKINSON, BROWN & GESELL 1940 GESELL & ATKINSON 1943)

The importance of the external and internal intercostal muscles to respiration has also posed a problem According to Hamberger s theory the external intercostal muscles and the intercartilaginous portion of the internal muscles are inspiratory and the interosseus portion of the internal muscles expiratory (cf CAMPBELL 1958)

Electrical recordings from the intercostal nerves (BRONK & FERGUSON 1935) and muscles (ANDERSON & LINDSLEY 1935) in cats showed the

external muscles to be inspiratory, and internal intercostal muscles expiratory. A similar finding was made by CHENNELS (1957) in experiment on both cats and rats. GESELL (1936) has shown that there are numerous individual variations in dogs. CAMPBELL (1955 a) has studied young men electromyographically with both surface and needle electrodes and has reported that in most male subjects the intercostal muscles of the lower intercostalspaces (5 — 9) contract during quiet inspirations. GREEN & HOWELL (1959) arrived at the same conclusion. TAYLOR (1960) on the basis of electromyogram studies with fine needle electrodes found the human external intercostal muscles to be purely inspiratory in action whereas the internal layer is expiratory except in regard to the parasternal part which is inspiratory. According to him in quiet inspiration only the parasternal region of the internal intercostal muscle is active but the external intercostal in the supine position is not. KOEPKE et al. (1955) who employed needle electrodes and an oscilloscope have stated that only in the first intercostal muscle is there electrical activity in quiet breathing; they were unable to separate the various layers of the intercostal muscles. JONES, BEARGIE & PAULY (1953), and JONES & PAULY (1957), who used surface electrodes for examination of muscles in the parasternal and mid clavicular regions discovered no rhythmic electrical activity in the intercostal muscles during quiet breathing. TOKIZANE et al. (1952) who used needle electrodes reported an absence of rhythmic electrical activity in the four upper intercostal muscles during quiet breathing. With a deepening of inspiration they detected a clear increase in the electrical activity of the intercostal muscles (JONES et al. 1953, CAMPBELL 1955 a, JONES & PAULY 1957, DRAPER, LADEFOGED & WHITTERIDGE 1957, KOEPKE et al. 1958, TAYLOR 1960).

With respect to animals it has been possible to demonstrate that the gradation of the contraction of intercostal muscles is dependent upon the recruitment of motoneurones, their action frequency and the action time (BRONK & FERGUSON 1935, GESELL, ATKINSON & BROWN 1941, GESELL, HUNTER & LILLIE 1949). The same mechanism is involved in regulation of the contraction of the diaphragm (GESELL et al. 1941, PITTS 1942). Nevertheless some research workers have stated that the synchronization of motor impulses is important to regulation of the contraction in the muscles of the diaphragm (DITTLER 1910, WINTERSTEIN 1911, DITTLER & GARTEN 1912, GASSER & NEWCOMER 1921, GASSER 1928, ADRIAN & BRONK 1928, HESS & WYSS 1936, Bishop & BLONSTEINBRENNER 1936, PIJLANT 1937), although many of them are of opinion that impulse synchronization occurs only with increasing

dyspnoe (DITTLER 1910, GASSER 1928, ADRIAN & BRONK 1928, WYSS 1939). WACHOLDER & MCKINLEY (1929) have stated that no synchronization exists in regulation of the contraction of the diaphragm.

Although extensive studies have been made of the electrical activity of the respiratory muscles in animals, relatively few research workers have compared the mechanical activity with the electrical activity measured either by integration of the action potentials or by counting the impulses from electromyograms. FINK, NGAI & DUNCAN (1958) have shown in the decerebrate cat that the integrated electromyogram activity of the diaphragm increases approximately linearly with the inspiratory load. RAMOS & ARJONA (1956) with Nembutal anaesthetized rabbits found that the »breathing activity« measured by mechanical or electrical integration of the electromyograms of the diaphragm bears a linear relationship to oxygen consumption. They determined the oxygen consumption with a respirometer of Collins type, the electromyogram of the diaphragm was registered with small steel serrefine electrodes fixed through the abdominal cavity on to the lower surface of the diaphragm. WOLDRING (1965) using anaesthetized cats with open thorax studied the activity of the diaphragm and abdominal muscles as a function of lung volume and arterial CO tension; intramuscular electrodes and electronic integration of action potentials were applied. He demonstrated that the integrated electrical activity of the diaphragm bears a linear relationship to the arterial CO tension when the lung volume is the independent variable, and moreover that the relationship between the diaphragm activity and the lung volume at a given arterial CO_2 tension is given by a straight line with a negative slope. GILL (1963) examined the effect of the end tidal CO on the discharge of phrenic motoneurons in decerebrated cats, and found that both the recruitment of additional units and the increase in discharge of phrenic units already active play a role in augmenting the phrenic discharge as the end tidal CO is increasing. BERGSTROM & KERTTULA (1961) with narcotized rats have shown that the inspiratory work determined by recording the changes in the inspired air volume and in the intrapleural pressure of the system in the course of inspiration bears a linear relationship to the impulse frequency of the electromyograms measured at the end of the inspiration. They also demonstrated that the total number of action potentials is in linear relationship to the first time integral of the inspiratory work. The electromyogram was registered with platinum thread brush electrodes and needle electrodes from the external intercostal muscles with the aid of a cathode ray oscillograph and amplifier.

In many human experiments electromyograms from the respiratory

muscles have been registered simultaneously with some mechanical functions of respiration, although comparatively few studies have been concerned with numerical evaluation of the relationships existent between the electrical and mechanical activity of the respiratory muscles. ROSSIFF et al. (1956) were probably the first to register an electromyogram from the diaphragm; they made use of needle electrodes and recorded rhythmic electrical activity, which increased during inspiration and almost totally vanished during expiration. AGOSTONI, SANT'AMBROGIO & DEL PORTILLO CARRASCO (1960) employed an oesophageal bipolar electrode for study of the electrical activity of the vertebral part of the diaphragm in man, and registered simultaneously the spirogram and/or the intrathoracic and gastric pressure. They found that during inspiration the electrical activity of the diaphragm was »approximately related to the transdiaphragmatic pressure as the activity found during graded inspiratory efforts at relaxation volume». By application of almost the same methods as AGOSTONI et al. (1960), PETIT, MILIC-EMILI & DELHEZ (1960) also found synchronization of the diaphragmatic activity with the respiratory variations of transdiaphragmatic pressure.

DELHEZ, PETIT & MILIC-EMILI (1959) (cf. PETIT, DELHEZ & TROQUET 1965) have shown that the integrated electromyogram activity of the recti abdomini in man is directly proportional to the calculated tension of abdomen muscles. DELHEZ et al. (1965) and DELHEZ, PETIT & BOTTIN (1965) have reported results from human experiments that the integrated electromyogram activity of the diaphragm bears a linear relationship to the calculated inspiratory work. The electromyogram from the diaphragm was recorded with oesophagus electrodes during individual inspirations and the total inspiratory work was calculated from dynamic, pulmonary elastic and thoracic elastic works.

CAMPBELL & GREEN have demonstrated that electromyogram activity registered with surface electrodes from the external oblique (1953 a, b) and rectus abdominis muscles (1953 a) is directly proportional to the intra-abdominal pressure during voluntary graded expiration when the chest volume remains constant. GRONBECK & SKOUBY (1960) with healthy males and patients with chronic asthma and emphysema have examined the electromyogram activity of the diaphragm with oesophagus electrodes; they found that during quiet breathing the inspiratory diaphragmatic discharge was generally greater in patients than it was in healthy persons, and further that the diaphragmatic activity definitely increased in the healthy persons when forced breathing was carried out.

MATERIAL AND METHODS

Material

The experimental subjects were 14 young, healthy males aged from 20 to 24 years As electromyogram recordings were made by means of surface electrodes persons with thick subcutis were excluded The electrical activity of the intercostal muscles and the mechanical activity of the inspiratory muscles were registered simultaneously during voluntary inspirations Analysis was made of 1055 voluntary inspirations of which 141 were registered during 5 ± 0 4% CO stimulation and 155 after it In addition 1033 voluntary inspirations were registered for determination of the alveolar relaxation pressure as a function of volume

Recording of electrical activity

The electrical activity of the intercostal muscles was registered electromyographically The recordings were made in a Faraday cage in a quiet room The electromyograms were generally registered with bipolar surface electrodes The electrode distance was 25 mm and the diameter of an electrode 7 mm The ordinary electrodes were pieces of felt clamped on plastic and fixed with Tensoplast to the skin which had been cleaned with ether Before the experiments the felt was moistened with electrode fluid (Mingograph electrode liquid Elema — Schönander) The choice of this type of electrode as the most suitable followed examination of a large number of surface electrodes For determination of the effect of the summation and subtraction of the action potentials in this electrode on the number and discharge frequency of electromyogram impulses simultaneous registrations with a DISA concentric needle electrode (diameter 0 35 mm) were made from the same intercostal muscle the two electromyograms were mutually compared From elec

amplifier integrator

diff manometer

pneumotachograph head

volume

flow

Wheatstone bridge

thoracometer

SUBJECT

electrodes

preamplifier

pressure

diff manometer

polygraph

amplifier

camera

oscilloscope

amplifier

Fig 1 a

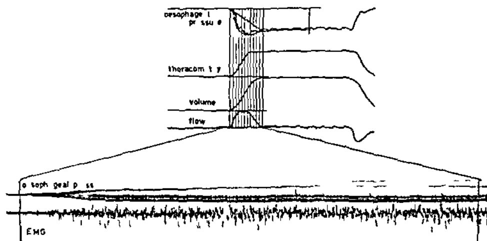


Fig 1 b

Fig 1 a) recording system

b) example of the various simultaneous registrations during experiments (tidal volume 2.50 l duration of inspiration 2.70 sec oesophageal pressure change measured at the end of inspiration 13.1 cm H_2O)

trodes the electromyogram impulses were led to a Tonnies differential preamplifier on to a Tetronix 502 oscilloscope and were filmed from the screen with a Physiologic Milano (Arduine) camera The gain of the pre amplifier was 100, and the sensitivity of the oscilloscope generally 1 cm/100 mV The speed of the film was 152 mm/sec For timing of the electro myogram with the recordings of respiratory movements the oesophagus pressure was led simultaneously to the other oscilloscope channel from which the beginning of each inspiration could be seen In view of the impossibility of determining the total number of action potentials in the action currents of all the motoneurons of the intercostal muscles recruited during inspiration a »sample» was taken from the electrical activity of muscles this was usually taken from the sixth intercostal muscle near the right mid clavicular line as during the preliminary investigations this region had proved to be the most satisfactory for recordings (VILJANEN et al 1965) The subjects were in a supine position during examination The size and position of the electrodes were kept constant, as were the amplification of the action potentials and other experimental conditions It can be assumed that a sample of the electrical activity of the intercostal muscles obtained in this way represents the total electrical activity (BERGSTROM & KERTTULA 1961) The following determinations were made by visual counting from the film 1) the total number of electromyogram impulses (n number) during inspiration 2) the electromyogram impulse frequency during a 1/3 second interval at the end of inspiration, in this study entitled the impulse frequency of dynamic phase (f_d) and 3) the electromyogram impulse frequency determined four seconds after the end of inspiration the subject holding his breath with the glottis open in this study entitled the impulse frequency of the static phase (f) 4) the increase in the number of electro myogram impulses during one and the same inspiration calculated as a function of time for comparison with the mechanical activity of respiratory muscles also measured as a function of time

The electromyogram impulses were counted by two persons and a third checked the counting by arbitrarily selected samples The differences between the results counted by two persons were 3 ± 1% From the electromyogram there were counted all the impulses which were distinguishable from the baseline irrespective of their location and amplitude In principle the method of counting was the same as that used by BERGSTROM (1959 a)

The mechanical activity of inspiratory muscles was determined from the alveolar relaxation pressure ($P_{a(\ )}$), the intra oesophageal pressure (P) and the inspiratory tidal volume (V̇) The movements of the thoracic cage were also registered

Measurement of tidal volume

The recording of the inspiratory volume (V) and flow (V̇) was effected with a Fleischpneumotachograph head (cf FLEISCH 1925 1956) No 294 (in which 5 mm H O equals 1 60 l/sec and 10 mm H O equals 3 19 l/sec) an Elema Schonander differential manometer EMT 572 No B 111 (range 0 50 mm H O), an electrical integrator Type EMT 573 No B 111 and a four channel polygraph Mingograph 42 In each experiment the volume was calibrated with a piston pump of known volume

Measurements of alveolar relaxation and oesophageal pressure

The alveolar relaxation and intra oesophageal pressure were re gistered with a Sanborn's differential pressure transducer model 270, the differential pressure range being $\pm$ 400 mm H_2O

Determination of the alveolar relaxation pressure was made by getting the subject under normal conditions to inspire a given volume and then to relax as completely as possible against an obstructed airway (ROHRER 1916 PAHN et al 1946) The volume and pressure were led to the poly graph and measured manually from the paper For each subject an average of 74 $\pm$ 19 pressure volume points was registered and the alveolar relaxation pressure ($P_{a(\ )}$) was determined from the c as a function of the volume (V)

The intra oesophageal pressure (P) was measured with an air containing latex balloon connected through the nose by a catheter to the manometer (BUYTENDIJK 1949 FRY et al 1952 MEAD et al 1955 CRANE HAMILTON & AFFELDT 1956 PETIT & MILIC EMILI 1958 SCHILDER HYATT & FRY 1959) The balloon was 10 cm in length the perimeter was 24 mm and the thickness of the wall of the balloon was approximately 0 15 mm the length of the polyethylene catheter 60 cm and the internal diameter 2 5 mm In the experiments there was 0 25—1 0 ml of gas in the balloon as it has proved that the error of measurement is small within this range (PETIT & MILIC EMILI 1958) During measurement the

balloon was in the lower third of the oesophagus (MILIC EMILI MEAD & TURNER 1964)

A water manometer was employed for pressure calibration during each experiment

Measurement of movements of the thoracic cage

Movements of the thoracic cage were measured with a rubber tubing of 2 0 mm bore and 3 5 mm external diameter wrapped around the chest The tube was filled with saturated $CuSO_4$ liquid The lengthening and narrowing of the column of $CuSO_4$ liquid increased its electrical resistance which formed one arm of a balanced Wheatstone bridge, when the re sistance varied it changed the balance of the bridge The changes were amplified and recorded with the polygraph The method is in principle the same as that applied by WADE (1954) The linearity of this transducer was good within the 0—10 cm range (in 50 measurements the linear correlation coefficient (*r*) was 0 998)

Calculation of the mechanical activity of the inspiratory muscles

The inspiratory muscles work against the following forces expressed in terms of pressure (ROHRER 1915 1916 MEAD 1961 for symbols cf MEAD & MILIC EMILI 1964) 1) the elastic forces of the lung and chest

$$P_{el(\ )} = P_{\ (l)} + P_{el(w)}$$

in which $P_{\ (l)}$ is the elastic force of the lung and $P_{\ (w)}$ is the elastic force of the chest 2) the flow resistive forces ($P_{res(\ )}$) which are dependent on the resistance of gas flow ($P_{r(g)}$) and on the non elastic resistance of the tissue deformation ($P_{\ (l)} + P_{\ (w)}$)

$$P_{\ (\ )} = P_{\ (l)} + P_{\ (g)} + P_{res(w)}$$

where $P_{es(l)}$ is the resistance of the non elastic deformation of the lung and $P_{\ (w)}$ that of the chest 3) the inertial forces ($P_{in(\ )}$) which are dependent on the mass of the tissues and gases

$$P_{in(\ )} = P_{\ (l)} + P_{in(g)} + P_{\ (w)}$$

where $P_{\ (l)}$ is the inertial force of the lung $P_{in(g)}$ that of the gas and $P_{\ (\ )}$ that of the chest

The total force of the inspiratory muscles (P_{mus}) is the equivalent of

the sum of the elastic forces flow resistive forces and inertial forces The equation of the total force of the inspiratory muscles is

$$P_m = P_{(\ )} + P_{(\ )} + P_{(\ )}$$

A large number of studies of normal lungs have indicated that the elastance is independent of breathing frequency (MEAD & WHITTENBERGER 1953 MEAD et al 1955, CHERNIACK 1956 OTIS et al 1956, RAU et al 1957 BUTLER & SMITH 1957 DEFARES & DONLEBEN 1960) This means that the alveolar relaxation pressure can be assumed to be equal to the total inspiratory elastic forces ($P_{el(\ )}$)

The flow resistive and inertial forces were determined by measurement of the intra oesophageal pressure as in many studies the changes in the intra oesophageal pressure correspond rather well to changes in the intra pleural pressure at least in the upright posture of the human being (DORNHORST & LEATHART 1952 FRY et al 1952 McILROY, MARSHALL & CHRISTIE 1954 ATTINGER MONROE & SEGAL 1956 BUTLER & SMITH 1957 FERRIS MEAD & FRANK 1959 KNOWLES HONG & RAHN 1959 MEAD & GAENSLER 1959 EHRNER 1960) In subjects studied supine the oesophageal pressure became less sub atmospheric than the intra pleural pressure it is concluded that this is attributable to oesophageal compression of the mediastinal structures in the supine posture (MEAD & GAENSLER 1959 KNOWLES et al 1959 FERRIS et al 1959) In the present study the changes in oesophageal pressure were measured in supine sub jects Nevertheless it is probable that the error in measurement is small since only the flow resistive and inertial forces of the lung were measured

For each inspiration the flow resistive and inertial forces of the lung were measured manually from the polygraph paper The increase in pressure was calculated as a function of time as follows the beginning and end points of the pressure curve were connected and this distance between this straight line and the actual pressure curve was measured at 0 25 sec intervals In principle this is the same method as that applied by NEERGAARD & WIRZ (1927) for their measurements of the intra pleural pressure The calculated flow resistive and inertial forces of the lung in each inspiration were added to the respective alveolar relaxation pressure and the sum of the two pressures as a function of the time was written as equal to the forces against which the inspiratory muscles work during inspiration In pursuance of this procedure the flow resistive and inertial work effected against the tissues of the thorax and abdomen is not included However this work is comparatively little when very forced inspirations are avoided (OTIS FENN & RAHN 1950 CHRISTIE 1953 AGOSTONI 1961)

If there is written

$$P_{dy} = P_{es(1)} + P_{in(1)} + P_{es(g)} + P_{i\,(g)}$$

the total force of inspiration muscles (P_{mu}) can be expressed as the following equation

$$P_m = P_{i(\ )} + P_{dyn}$$

In each inspiration pressure P_m was determined as a function of time ($P_{mu} = f(t)$) for the mathematical manipulation

When the force of inspiratory muscles (P_m) and the change of volume in the lung during inspiration are known as functions of time there can be calculated 1) the inspiratory muscular work (W) during breathing which is

$$W = \int P_m \times dV ,$$

2) the first time integral of the inspiratory muscular work (H_t) by application of the equation

$$H_t = \int W \times dt ,$$

and 3) the pressure impulse of the inspiratory muscles (I) by application of the equation

$$I = \int P_{mu} \times dt$$

all as functions of time These can be related to the respective electrical activity of the intercostal muscles also expressed as a function of time

In each inspiration the changes in pressure and volume were measured from the polygraph paper at intervals of 0 25 seconds A computer (μ Linc) was employed in calculation of the equations Thus the following parameters were obtained 1) tidal volume (V) 2) the force of the inspiratory muscles (P_{mus}) 3) muscular work (W) 4) the first time integral (H) of the muscular work and 5) pressure impulse (I) all in digital form at 0 25 seconds intervals as a function of time

CO_2 stimulation

In the CO stimulation experiments the six subjects breathed in through a flap valve from a plastic bag containing 2000 litres 5 ± 0 4% CO_2 air and expired through another valve into the athmospheric air The flow resistance of the valve was 1 litre/sec corresponding to 15 mm

H_2O and 1 5 litres/sec corresponding to 31 mm H_2O The EMG from the external intercostal muscles, and the corresponding mechanical magnitudes of the normal state were recorded before CO_2 stimulation was started When stimulation had continued for 25 ± 9 min, similar registrations were made again The recording period was 12 ± 2 min Immediately after this CO_2 stimulation was discontinued by switching the valve to normal athmospheric air These registrations were repeated after 14 ± 2 min During the whole course of the experiment the position of the electrode the necessary amplifications and other experimental conditions were kept constant The subjects in this experiment were also in a supine position

Experimental conditions

The experiments were carried out in a quiet room and no recordings were made before the subjects had rested for at least half an hour The experiments for measurement of the alveolar relaxation pressure lasted 1—1½ hours and those in which the electrical and mechanical activities during inspiration were measured simultaneously continued for 1½—2½ hours To avoid possible hypocapnia in the blood there were 5—10 quiet inspirations between the voluntary inspirations and also 2—3 pauses 5 minutes in duration In the experiments endeavours were made to minimize fatique, particularly in view of the following points raised by various researchers 1) it has been shown that when a skeletal muscle fatigues greater integrated electrical activity is needed to effect a given tension than in a rested muscle This applies to both isometric (EDWARDS & LIPPOLD 1956) and anisometric contractions (SCHERRER SAMSON & SOULA 1954) 2) the effect of fatigue on the impulse frequency is not clear some research workers feel that no change occurs (LINDSLEY 1935 BIGLAND & LIPPOLD 1954 b) have reported a diminution in the frequency (COBB & FORBES 1923), or have observed an increase in the incidence of polyphasic potentials (BUCHTAL PINELLI & ROSENFALK 1954) 3) exercise has been found to reduce the impulse frequency (JALAVISTO et al 1938 JALAVISTO KERANEN & SEPPALA 1939 STEPANOV 1959)

In all the experiments the subjects were in a supine position Throughout series of 6—8 inspirations were effected, with the volume increased at each inspiration (e g 0 5 1 0 1 5 2 0 4 0 l) 10—15 series were registered

Statistical methods

The standard deviation (s) was calculated by means of the usual formula

$$\hat{s} = \frac{\sqrt{N \sum x - (\sum x)}}{N - 1}$$

where x is a variable and N the number of observations

The coefficient of linear correlation (r) was arrived at from

$$r = \frac{s_{xy}}{s_x \, s_y},$$

where s_{xy} is the covariance of variables x and y, calculated from

$$s_{xy} = \frac{1}{N - 1}\left[\sum xy - \frac{\sum x \sum y}{N}\right]$$

The standard deviation of x s_x was found from

$$s_x = \sqrt{\frac{1}{N - 1}\left[\sum x - \frac{(\sum x)}{N}\right]}$$

and the standard deviation of y s_y from

$$s_y = \sqrt{\frac{1}{N - 1}\left[\sum y - \frac{(\sum y)}{N}\right]}$$

The regression coefficient was calculated

$$b = \frac{s_{xy}}{s}$$

and the point at which the regression line meets the y axis

$$c = \frac{\sum y \sum x^2 - \sum x \sum xy}{N \sum x^2 - (\sum x)}$$

The modified standard error of estimate which measures the concentration of the points about the line of regression was calculated from

$$s_{y \cdot x} = s_y \sqrt{1 - r} \sqrt{\frac{N - 1}{N - 2}}$$

The existence of linear stochastic dependence was subjected to a t test The coefficient t was arrived at from

$$t = \frac{r\sqrt{\lambda - 2}}{\sqrt{1 - r}}$$

where the number of degrees of freedom is $\lambda - 2$

The significance of the difference between two regression coefficients from independent regression analyses was subjected to a t test

$$t_b = \frac{b_1 - b_{-}}{\sqrt{\frac{s_{y\ x}^{(1)2}}{(\lambda_1 - 1)\, s_x^{(1)}} + \frac{s_{y\ x}^{(\ )}}{(\lambda_{-} - 1)\, s_x^{(\ )2}}}}$$

where the number of degrees of freedom was calculated for simplicity as $N_1 + N - 2$

The significance of the difference between two constant terms (c) was subjected to a t test

$$t = \frac{c_1 - c_{*}}{\sqrt{s_{c_1} + s_{\ *}}}$$

where

$$s_c = \frac{\sum x \quad \hat{s}_y}{N \sum x - \left(\sum x\right)}$$

and the number of degrees of freedom is $N_1 + N$

The mean of the coefficients corre s fc means of the transformation

$$\sum_1^m$$

$$\sum_1^m$$

where

and

the latter being the st

The references are W

RESULTS

Experiments under normal conditions

Electromyogram impulse frequency at the end of inspiration and inspiratory muscular work

The impulse frequency recorded from the external intercostal muscles during a 1/3 second interval at the end of inspiration what is known as the impulse frequency of the dynamic phase (f_d) bears a linear relationship to the inspiratory muscular work (W) during voluntary inspiration within a certain range (Fig 2) Fig 2 illustrates the result calculated from 42

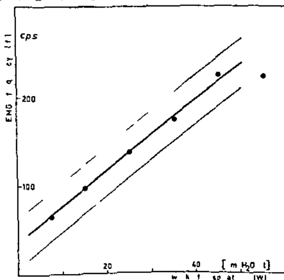


Fig 2 Electromyogram impulse frequency (f_d) at the end of inspiration as a function of inspiratory muscular work (W) The points represent the mean of at least 6 values of the variables in columns with a class interval of 10 cm $H_2O \times$ l There are 42 values of the variable in the range 0–50 cm $H_2O \times$ l the regression coefficient (b_d) is 4 0 cm $H_2O^{-1} \times sec^{-1} \times l^{-1}$ and the constant (c_d) 38 6 cps the coefficient of the linear correlation (r) 0 90 and the modified standard error of estimate ($\bar{s}_y$) ± 9 cps

values of the variables within the range 0—50 cm H_2O × l the regression coefficient (b_d) is 4 0 cm H $O^{-1} \times sec^{-1} \times l^{-1}$ and the constant (c_d) 38 6 sec^{-1}, the coefficient of the linear correlation (r) 0 90 the t test value (t_r) 13 1 and the modified standard error of estimate ($\hat{s}_{y\,x}$) ± 27 9 sec^{-1} The points represent the mean of at least 6 values of the variables *in columns with a class interval of 10 cm H O × l The volume of the* smallest inspiration included is 0 58 l the corresponding muscular work is 4 4 cm H O × l and the volume of the largest inspiration 2 74 l with the representative inspiratory muscular work 47 9 cm H_O × l The mean of the upper limits of the linear range in the 14 experimental subjects was 44 ± 10 cm H O × l The volumes of the inspirations varied between 0 4—3 6 litres The linearity can be expressed by the equation

$$f_d = b_d W + c_d ,$$

where b_d is the regression coefficient, and c_d the constant of the line The arithmetic mean (M_{b_d}) of the regression coefficients was 5 1 ± 2 2 cm H $O^{-1} \times sec^{-1} \times l^{-1}$ and the mean of the constants (M_{c_d}) 25 3 ± 30 3 sec^{-1} (Table 1) The mean of the linear correlation coefficients (r) determined by the z transformation was 0 90 The detailed findings in respect of the individual subjects are listed in Table 1 of the Appendix which also includes the t test values (t) and the modified standard errors of estimates ($\hat{s}_y$) The corresponding regression lines are indicated in Fig 3

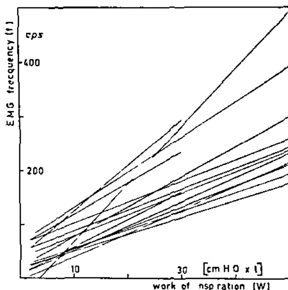


Fig 3 Electromyogram impulse frequency (f_d) at the end of inspiration as a function of the inspiratory muscular work (W) from 14 experimental subjects separately

TABLE 1

MEAN VALUES OF REGRESSION COEFFICIENTS (M_b) AND CONSTANTS (M) DERIVED FROM EQUATIONS $n = b_n H_t + c$ $f_d = b_d W + c_d$ $f = b P_{( )} + c$ IN RESPECT OF 14 PERSONS where n is total number of electromyogram impulses H_t the first time integral of inspiratory muscular work f_d the electromyogram impul e fr quency at the end of inspiration W inspiratory muscular work f the electromyogram impulse frequency four seconds after the end of inspiration with the subject holding his breath b b_d and b are regression coefficients and c c_d and c the corresponding constants The mean of the regres ion coefficients ($\bar{r}$) was determined by means of the trans formation

Number of subjects	Number of inspirations N	Mean of regres ion coefficients M_b $cmH_2O^{-1} \times sec^{-1} \times l^{-1}$	Mean of constants M_n	Standard deviation $\|\delta\|$	Mean of correlation coefficients $\bar{r}$	Number of inspirations N	Mean of regression coefficients M_{b_d} $cmH_2O^{-1} \times sec^{-1} \times l^{-1}$	Mean of constants M_d sec^{-1}	Standard deviation $\|\delta\|$	Mean of correlation coefficients $\bar{r}$	Number of in pirations N	Mean of regression coefficients M_b $cmH_2O^{-1} \times sec^{-1}$	Mean of constants M sec^{-1}	Standard deviation $\|\delta\|$	Mean of correlation coefficients $\bar{r}$
14	337	7 6	46 3	2 0 44 3	0 91	92	5 1	25 3	2 2 30 3	0 90	359	13 1	−38 7	3 7 39 6	0 91

Total electromyogram impulse number and the first time integral of the inspiratory muscular work

The total number of the electromyogram (n) registered from the external intercostal muscles during inspiration is linearly proportional to the first time integral (H_t) of the inspiratory muscular work within a certain range In Fig 4, the total impulse number (n) of the electro

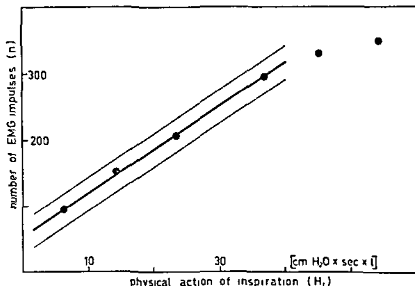


Fig 4 Total number (n) of electromyogram impulses as a function of the first time integral (H_t) of the inspiratory muscular work The points represent the mean of at least 6 values of the vairables in columns with a class interval of 10 cm H_2O × sec × l There are 41 values of the variables in the range 0—40 cm H_2O × sec × l the regression coefficient (b) is 6 7 cm H_2O^{-1} × sec^{-1} × l^{-1} and the constant of the regression line (c) 51 1 the coefficient of the linear correlation (r) 0 94 and the modified standard error of estimate ($\hat{s}_{y\ x}$) ± 25 4

myogram is given as a function of the first time integral (H_t) *of the* muscular work the »physical action» (dimension cm H O × sec × l comp BERGSTROM 1962) The results relate to the same subject as above The figure contains 41 points in the H_t range 0 to 40 cm H O × sec × l the regression coefficient (b) is 6 7 cm H_2O^{-1} × sec^{-1} × l^{-1} and the constant term (c) 51 1 the coefficient of the linear correlation (r) is 0 94 the value of the t test (t) 17 2 the modified standard error of estimate ($\hat{s}_y$) ± 25 4 The points indicate the mean of at least 6 values of the variables in the columns in which the class interval is 10 cm H O × sec × l The smallest inspiration included is 0 58 l and its physical action (H_t) 1 8 cm H O × sec × l The deepest inspiration within the range 0—40

cm H_2O × sec × l is 2 84 l its H_t = 39 9 cm H O × sec × l It thus becomes possible to combine the results in the equation

$$n = b\ H_t + c$$

The arithmetical mean of the regression coefficients (M_{b_n}) from the 14 experimental subjects is 7 6 ± 2 0 cm H_2O^{-1} × sec^{-1} × l^{-1} and the arithmetical mean of the constants (M_{c_n}) is 46 3 ± 44 3 The mean of the linear correlation coefficients (r) calculated applying the z transformation is 0 91 (Table 1) The mean value of the upper limits of the linear ranges in the 14 subjects was 53 ± 10 cm H O × sec × l The findings in respect of the individual subjects are given in Table 1 of the Appendix, and the regression lines in Fig 5

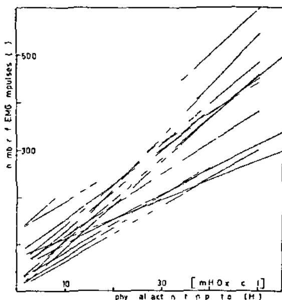


Fig 5 Total number (n) of the electromyogram impulses as a function of the first time integral (H_t) of the inspiratory muscular work from 14 experimental subjects separately

Electromyogram impulse frequency four seconds after the end of inspiration and the alveolar relaxation pressure

The impulse frequency of the electromyogram, determined four seconds after the end of the inspiration with the subject holding his breath with the glottis open the so called »impulse frequency of the static phase (f)» bears a linear relationship to the corresponding alveolar

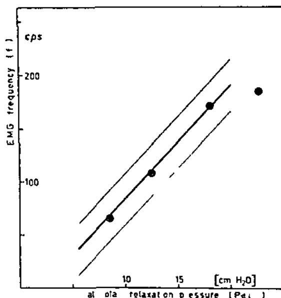


Fig 6 Electromyogram impulse frequency four seconds after the end of inspiration the subject holding his breath (f) as a function of the alveolar relaxation pre ure ($P_{st(\ )}$) The points represent the mean of at least 6 values of the variables in columns with a class interval of 5 cm H_2O There are 39 values of the variables within the range 0 – 20 cm H_2O the regression coefficient (b) is 10 8 cm $H_2O^{-1} \times sec^{-1}$ and the constant of the regression line (c) — 24 ɷ cp the coefficient of the linear correlation (r) 0 78 and the mo lified stan dard error of estimate ($\hat{s}_y$) $\pm$ 22 0 cps

relaxation pressure ($P_{(\ )}$) (Fig 6) This again can be expressed by an equation

$$f = b\ P_{(\ )} + c$$

where b is the regression coefficient and c the constant term Fig 6 relates to the same subjects as above In the figure the regression line is calculated from 39 values of the variables within the range 0—20 cm H O the regression coefficient (b) is 10 8 cm $H_2O^{-1} \times sec^{-1}$ and the constant term (c) is 24 5 sec^{-1} the coefficient of the linear correlation (r) 0 78 the value of the t test (t) is 7 6 and the modified standard error of estimate ($\hat{s}_{y\ x}$) $\pm$ 22 0 sec^{-1} The points are the mean of at least 6 values of the variables in columns with a class interval of 5 0 cm H O The smallest inspiration included is 0 41 l and its alveolar relaxation pressure 5 1 cm H O The deepest inspiration in the range 0—20 cm H O is 2 46 l and its alveolar relaxation pressure 19 6 cm H O

The arithmetical mean of the regression coefficients (M_b) in respect of the 14 experimental subjects is 13 1 $\pm$ 3 7 cmH $O^{-1} \times sec^{-1}$ and that of the constant terms (M) — 38 7 $\pm$ 39 6 sec^{-1} The mean of the linear

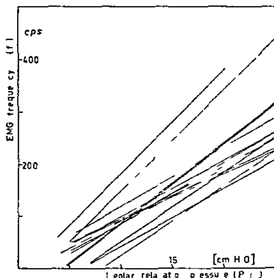


Fig 7 Electromyogram impulse frequency (f) four seconds after the end of inspiration the subject holding his breath as a function of the alveolar relaxation pressure (P ( )) from 14 experimental subjects separately

correlation coefficient (r) (z transformation) is 0 91 (Table 1) The mean value of the upper limits of the linear range is 24 ± 3 cm H O The findings with respect to the individual subjects are present in Table 1 of the Appendix and the corresponding regression lines in Fig 7

Electromyogram impulse number and the pressure impulse produced by inspiratory muscles

In Figure 8 the electromyogram impulse number (n) during one and the same inspiration is plotted against the corresponding pressure impulse produced by the inspiratory muscles ($I = \int P_m \wedge dt$ where t is the duration of the inspiration) It is observable that there exists a linear relationship between these two variables These can be combined in the equation of the line

$$n = b\ I + c$$

where b is the regression coefficient and c the constant term The slope of these variable varies in different kinds of inspiration its steepness is determined at the beginning of inspiration The arithmetical mean of the coefficient of the linear regression calculated from 100 inspirations recorded from ten different subjects was 0 998 The results

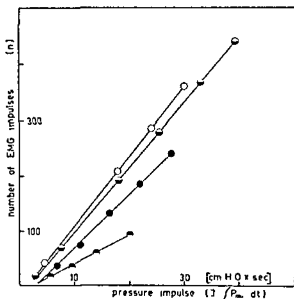


Fig 8 Number of electromyogram impulses (n) as a function of the pressure impulse (I) produced by the inspiratory muscles during one and the same inspiration The figure relates to four different inspirations

obtained from six subjects are given in Tables 3 a—3 f of the Appendix This also means that during the deepening of the inspiration the impulse frequency of the electromyogram registered from external intercostal muscles is directly proportional to the pressure produced by the inspiratory muscles (P_{mus}) although the slope varies after different inspirations

Experiments during CO_2 stimulation

Registration from six experimental subjects were taken before during and after 5 ± 0 4% CO_2 stimulation The regression lines of the linear correlation which represent the relationship of the electromyogram impulses (n) and the physical action (H_i) are shown in Fig 9 (○ — ○ — ○ = before ● — ● — ● = during ⊖ — ⊖ — ⊖ = after stimulation) The *regression lines are calculated respectively from 37, 8 and 10 inspirations* and the corresponding coefficients of the linear correlation are respectively 0 80 0 87 and 0 92 the regression coefficients 9 5 5 1 and 10 8 cm $H_2O^{-1} \times sec^{-1} \times l^{-1}$ the constant terms 124 2 146 2 and 96 8 and the modified standard errors of the estimates ± 120 7 ± 50 7 and ± 59 2 respectively Figure 9 illustrates that the slope of these variables is less

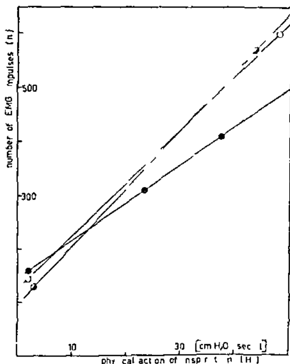


Fig 9 Total number of electromyogram impulses (n) as a function of the first time integral of the inspiratory muscular work (H_t) before (O—O—O) during (●—●—●) and after (◒—◒—◒) 5 0% CO_2 stimulation

during CO_2 stimulation than before and after it Table 2 contains the arithmetical means of the regression coefficients (M_{b_n}) and of the constant terms (M) and their standard deviations ($\hat{s}$) before (A) during (B) and after (C) stimulation There are further the means of the coefficients of the linear correlations (r) calculated by the z transformation which are 0 92 0 88 and 0 91 respectively Table 2 also gives the arithmetical means of the regression coefficients of equations $f_a = f(H)$ and $f = f(P_{()})$ and the corresponding mean of the coefficients of the linear correlations (r) (z transformation) The linearity is evident in these equations in the first case the values of r are 0 94 0 90 and 0 93 respectively and in the second 0 92 0 87 and 0 94

It is observable from Fig 9 Table 2 and Table 2 of the Appendix which contains the results in respect of individual subjects that the slope between the number of electromyogram impulses and the physical action is less during CO stimulation than before and after it although the t test indicates a statistical significant difference ($p < 0\ 01$) in only

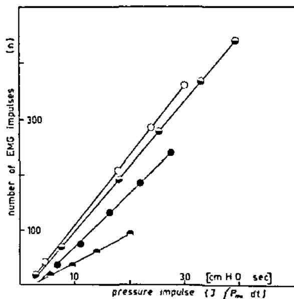


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one case in the course of transfer from A state to B state ($t_b = 2.75$) and in three cases from B state to C state ($t_b = 9.75$, 3.21, 3.02) (refer to Table 2 in the Appendix, t_b) Moreover the constant term (c) is always numerically greater during CO stimulation than before and after it although a satistical significant difference is apparent twice only (t = 3.18 and 3.36) In three instances the regression coefficient (b_d) between the impulse frequency of the dynamic phase (f_d) and the inspiratory muscular work (W) is significantly less during CO stimulation than before it ($t_b = 3.90$, 6.35 and 3.32) and in three instances less than after stimulation ($t_b = 4.14$, 5.09 and 5.60) With regard to constant (c_d) there is one statistical significant difference ($t = 2.88$)

To judge from the regression coefficient (b) between the electromyogram impulse frequency of the static phase (f) and the alveolar relaxation pressure (P_{rel}) in none of the cases is there any statistical significant difference this applies also to the corresponding constant (c) The findings in respect of the individual subjects are presented in Table 2 of the Appendix

During CO stimulation the linear correlation between the electromyogram impulse number (n) and the corresponding pressure impulse (I) remains The arithmetical mean of the coefficients of the linear correlations calculated from 60 inspirations recorded from six subjects was 0.998 The findings in respect of the individual subjects are contained in Table 3a—3f in the Appendix along with the results of post stimulation registration

Comparison of the electromyograms recorded with surface electrodes and needle electrodes

Fig 10 shows the impulse number (n) of the electromyogram taken from 48 inspirations recorded with concentric needle electrodes plotted against the electromyogram impulse number (n) recorded simultaneously with surface electrodes These variables are clearly in linear correlation The regression coefficient is 0.41 and the coefficient of linear correlation 0.98

The regression coefficient is the same 0.41 on comparison with the impulse frequency of the electromyogram registered at the end of the inspiration The coefficient of linear correlation is 0.96 The experiments were concerned with six experimental subjects and the correlation coefficient (r) varied between 0.95—0.99 ($p < 0.001$)

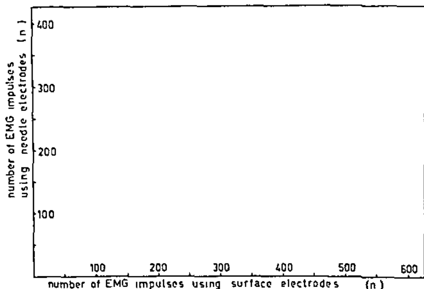


Fig 10 Electromyogram impulse number (n) recorded with concentric needle electrodes from intercostal muscles as a function of the electromyogram impulse number (n) recorded with surface electrodes from the same muscles *The regression coefficient is 0 41* and the coefficient of linear correlation (*r*) 0 98 calculated from 48 values of variables

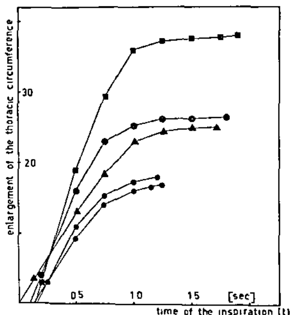


Fig 11 Enlargement of the thoracic cage as a function of time during five different inspirations The unit of enlargement is arbitrary

Movements of the thoracic cage in inspiration

Fig 11 illustrates a typical example of the enlargement of the [illegible] cage during inspiration as a function of time It is [illegible] thoracic cage is enlarged in different ways during different [illegible], and furthermore that the velocity at the beginning is rath[illegible] the same inspiration, and becomes slower at its end

DISCUSSION

Gradation of the voluntary contraction of the skeletal muscle in man is based upon recruitment of the motoneurons their action frequency, and action time (ADRIAN & BRONK 1929 SMITH 1934 LINDSLEY 1935 GILSON & MILLS 1941 TOKIZANE, KAWAMATA & TOKIZANE 1952, BIGLAND & LIPPOLD 1954 b) in regard to the skeletal muscles this has been demonstrated by TOKIZANE et al (1952) for the intercostal muscles specifically Similar gradation has been found in animals both in the intercostal muscles (BRONK & FERGUSON 1935, GESELL ATKINSON & BROWN 1941 GESELL HUNTER & LILLIE 1949) and in the diaphragm (GESELL et al 1941 PITTS 1942) BERGSTROM (1958 1959 b) reduced these three gradating factors to one alone, the number of action potentials of the set of motoneurons involved in the muscle contraction

Nevertheless it has been shown that the electromyogram impulses are equivalent to the nerve impulses fired into the muscle during the contraction (DITTLER 1910 DITTLER & GARTEN 1912 GASSER & NEWCOMER 1921) Consequently the electromyogram impulses also represent the corresponding nerve impulses

It is impossible to record the total electrical activity of the intercostal muscles All that can be done is to take a sample from the total electrical activity of an electromyographical registration as was effected by BERGSTROM & KERTTULA (1961) it can be assumed that the sample taken by a non selective technique represents the total electrical activity This means that these two bear a linear mutual relationship This is also supported by the linear relationship of the electromyogram activities recorded simultaneously by means of surface and concentric needle electrodes (Fig 10) Furthermore, BIGLAND & LIPPOLD (1954 b), on comparing surface electrodes with concentric needle electrodes observed that the integrated electrical activities from the extensor digitorum communis had a linear relationship at low activity levels in isometric contraction They concluded that addition and subtraction occur in

such a way that the final output remains constant DELHEZ & PETIT (1961) have shown that both methods of detection provide similar results They recorded electromyograms from human abdominal muscles from m sternocleidomastoideus and m scalenus and also from m trapetzius and m pectoralis major but pointed out a marked variability in the individual values for activity ANDERSSON & LINDSLEY (1935) have demonstrated that the external intercostal muscles of two sides of the thorax in normal and unilateral pneumectomized cats are activated equally both in regard to the number of units involved and the average frequency of the responses in each The single motor unit technique was applied GASSER & NEWCOMER (1921) recorded impulses from the right phrenic nerve and the left portion of the diaphragm observed that the discharges were symmetrical and decided that a common centre must regulate impulsation on both sides BOYD & BASMAJAN (1963) implanted multiple clip electrodes in the diaphragm of rabbits at open operation, and found no obvious general pattern of difference in the electric activity between various muscular slips of the same rabbit s diaphragm TAYLOR (1960) took simultaneous recordings from human diaphragm through a needle electrode at the costal margin and through oesophageal electrodes and discovered no real difference in the timing of the activity detected in the two sites

When the size and position of the electrodes and other experimental *conditions are kept constant it has been assumed in the present study* that with a non selective technique the electromyogram represents the total motor discharge fired by the nervous system into the inspiratory muscles during an inspiratory cycle as supposed by BERGSTROM & KERTTULA (1961) in regard to the intercostal muscles of rats This implies that at the same time as reference is made to the electrical and mechanical activity of the intercostal muscles there is reference to the relation between the electrical activity of the intercostal nerves and the mechanical activity of the inspiratory muscles

On comparison of the impulse frequency of the electromyogram recorded from external intercostal muscles at the end of inspiration and the inspiratory muscular work done in inspiration frequency (f_i) was found to have a linear relationship to the work (W) (Fig 2) The results given above have also shown that the total number of impulses counted from the electromyogram sample during inspiration is directly proportional to the first time integral of the inspiratory muscular work (H_i) the so called physical action (dimension cm H O × sec × l cf BERGSTROM 1962) (Fig 4) These results are in accord with findings in respect to the activity in the intercostal muscles of rats where also the electromyogram

impulse frequency taken at the end of inspiration is linearly proportional to the relative inspiratory work determined from the amplitude of the volume curve and from changes in the intra pleural pressure In these experiments it was further demonstrable that the total number of the action potentials determined from the electromyogram sample rose proportionally to the time integral of the respiratory work (BERGSTROM & KEPTTULA 1961)

In the main the same finding was made by BERGSTROM in his experiments with voluntary finger muscles of the human being the results indicate that the impulse frequency of the electromyogram is directly proportional to the degree of muscle shortening at different loadings of the contractions the amount of work produced by unloaded contraction and also to the estimated muscular work necessary to attain the degree of abduction (1959 c) He has further demonstrated that the number of electromyogram impulses bears a linear relationship to the physical action (1959 b)

From the results outlined above there can be written

$$n = b \cdot H_t + c$$

where n is the total number of action potentials H_t the first time integral of the work ($H_t = \int W \times dt$) b is the regression coefficient, and c the constant The right side of the equation is thus a function of time as is also magnitude n which involves the three gradating factors the number of the recruited motor units their action frequency and action time During inspiration the retraction force of the respiratory system tries to return the system to the functional respiratory level This entails that the longer the inspiration takes the greater the number of action potentials becomes during a given voluntary inspiration although the inspiratory work is not increasing with time This implies that the results reported by DELHEZ et al (1965) and DELHEZ PETIT & BOTTIN (1965), which indicate that the integrated electromyogram activity of the human diaphragm is directly proportional to inspiratory work are valid for these reasons only when the duration of inspirations is constant since the integrated electrical activity is a function of time but the work is not

Earlier it was shown that the electrical activity of the skeletal muscle bears a linear relationship to the muscle tension both in isometric (JALAVISTO et al 1939 LIPPOLD 1952) and in anisometric contraction (BIGLAND & LIPPOLD 1954 a) In principle this is also apparent in the finding that the impulse frequency of the electromyogram determined four seconds after the end of the inspiration with the subject holding his breath with the glottis open the impulse frequency of the static

phase (f)) is in linear relation to the corresponding alveolar relaxation pressure ($P_{a(\ )}$) (Fig 6) Here the contraction of the intercostal muscle is anisometric, but the velocity is zero The four second interval after the end of the inspiration was chosen in view of earlier experiments (VILJANEN et al 1965) In that phase the electromyogram activity of the intercostal muscles has reached almost a constant level after the inspiration moreover the subject can quite easily hold his breath for such a period

It is interesting to observe that during one and the same inspiration the electromyogram impulse number (n) is directly proportional to the force impulse of the inspiratory muscles, here expressed as the pressure impulse ($I = \int P_m \times dt$) (Fig 8) This means that during inspiration the electromyogram impulse frequency bears a linear relation to the pressure produced by the inspiratory muscles (P_m) The slope between the impulse number (n) and the pressure impulse (I) varies in different types of inspiration but the linear regression still remains If a start is made from the linear correlation of the electrical activity of the intercostal muscles and the pressure produced by the inspiratory muscles (P_m) it can be concluded that during the whole inspiration the relative quantity of the inspiratory muscles remains on a constant level with the total inspiratory muscular force (P_m) It is likely that in different inspirations variations occur in the proportion of the intercostal muscles which form the total inspiratory muscular force although it remains constant within one and the same inspiration It also seems that at the beginning of each inspiration the respiratory centres determine which muscles will take part in the inspiration and the extent of such participation (VILJANEN 1967 b) It should be pointed out that in deep inspirations in which the physical action is great the linearity between the total electromyogram impulse number (n) and the physical action (H) no longer applies (Fig 4) In each of these inspirations the electromyogram impulse number rises in a linear relation to the corresponding pressure impulse except as regards very forced inspirations

Accordingly in each inspiration with the exclusion of those which are very forced in nature the electromyogram impulse frequency is directly proportional to the pressure produced by the inspiratory muscles (P_m) Nevertheless at the end of the inspiration the electromyogram impulse frequency of the external intercostal muscle also bears a linear relationship to the inspiratory muscular work (Fig 2) This means that at the end of the inspirations the muscular force expressed as pressure (P_{ma}) and the muscular work (W) are in direct proportion according to the formal equation

$$P_m = a \times W = a \times \int P_m \times dV,$$

where a is a constant V = the tidal volume Consequently the force and the distance, and thus the pressure (P_m) and the volume (V) must have a logarithmic relation when other factors are constant Further, the impulse frequency of the intercostal muscle electromyogram at the end of inspiration (f_d) and the tidal volume (V), have a logarithmic relation when the other factors remain constant (VILJANEN 1966) This can be expressed by the equation

$$\ln f_d = \lambda_1 + \lambda \times V,$$

where λ_1 and λ are constants

Possibly the electromyogram impulse frequency during the whole inspiration would be in logarithmic relationship to the increase in tidal volume were it not for the increased firing of motor impulses from the muscular spindle systems stimulated by the flow resistive and inertial muscle tension, which are zero at the end of the inspiration The firing of motor impulses from the spindle systems has been demonstrated by CRITCHLOW & von EULER (1962) in cats

Furthermore it seems that the respiratory centres are programmed to find a determined end state in the inspiratory volume during each inspiration, reached also by the respiratory system if the peripheral conditions stay constant It might be that the pursuit of a given volume results from the sending of a determined nervous impulse train to the inspiratory muscles and that the physical action of the inspiratory muscle is determined by this means The number of impulses might then be one explanation for the »demand for tidal volume» in CAMPBELL s & HOWELL s (1962) hypothesis on the nervous regulation of respiration A possible explanation of the findings of CAMPBELL & his co workers (1961 a) and CAMPBELL DINNICK & HOWELL (1961) that a sudden increase of the inspiratory load is followed by a diminution in the tidal volume and overcome only progressively is that after the delivery of the impulse train the nervous centres can no longer correct the impulse number Moreover there exists the possibility that a sudden reduction in the load involves a corresponding deepening of the tidal volume In other words, with a given impulse number it is possible to release a certain amount of energy This is in accord with the findings of PAMOS & ARJONA (1956) that the electrical activity of the diaphragm bears a linear relationship to the oxygen consumption It could be said that from »experience» (plus additional other orders) the inspiratory centres know how many

nervous impulses are needed for an adequate tidal volume Under normal conditions this amount results in attainment of the tidal volume required Further, it also seems possible that the above is an example of a more general central nervous system control of the muscular function suggested by BERGSTROM (1958)

If the velocity of the respiratory system during one and the same inspiration is constant as it appears to be over a wide range at lea t (Fig 11) the electromyogram impulse number registered from the external intercostal muscle during one inspiration is in a linear relationship to the *inspiratory muscular work* since the *electromyogram impulse* number and the pressure impulse (I) bear a linear relation as well (Fig 8) At the end of the inspiration, this no longer applies, since there the increase in the volume is nearing zero as is consequently the inspiratory muscular work as well although electromyogram impulses are continuously discharded in order to balance the retraction force of the lung

During the course of $5\ 0 \pm 0\ 4\%$ CO_2 stimulation these relationships between the electrical and mechanical activity are valid The results obtained are in agreement with the findings of BERGSTROM & KERTTULA (1961) in regard to the intercostal muscles of the rat they demonstrated that during CO stimulation the linear relations still existed between the electromyogram impulse number registered from the external intercostal muscle of the rat during inspiration and the physical action as well as between the electromyogram impulse frequency at the end of the inspiration and the relative inspiratory work It is evident that in each experiment the slope of the linear regression between the number of the electromyogram impulses and the physical action is less during CO_2 stimulation than before and after it (Table 2, Table 2 of the Appendix) No more than a few experiments were conducted with CO_2 stimulation, in view of the discomfort which arises if 5% CO_2 air is breathed for an extended period This may explain the existence of statistical significant differences in some cases only A similar tendency was also apparent in the relationship between the impulse frequency of the dynamic state (f_d) and the inspiratory muscular work (W) (Table 2 and Table 2 of the Appendix) During the CO stimulation larger EMG impulse values were also registered at the beginning of inspiration although the differences were not statistical significant (Table 2 Fig 9)

It is interesting to note the diminution of the correlation coefficient in the CO_2 stimulation experiments this might be attributable to the rising end tidal CO_2 tension increasing the work output (MILIC EMILI & TYLER 1963) although some other factors may be implicated as well e g a smaller proportion of the intercostals being recruited

All the present recordings were made from human intercostal muscles The studies have been extended to the human diaphragm Preliminary findings indicate the likelihood of a similar relationship existing between the electrical and mechanical activities in the human diaphragm (VILJANEN 1967 a)

SUMMARY

1 A comparison has been made between the electromyographically registered activity of the intercostal muscles in man during voluntary inspiration and the corresponding mechanical activity of the inspiratory muscles i e the pressure and the pressure impulse produced by inspiratory muscles the inspiratory muscular work and the first time integral of the inspiratory muscular work calculated from the alveolar relaxation pressure oesophageal pressure and tidal volume the experiments were conducted both under normal conditions and during CO stimulation

2 The total electromyogram impulse number during the course of inspiration bears a linear relationship to the first time integral of the inspiratory muscular work the so called »physical action» However this linearity did not apply to either very forced or deep inspiratory excursions

3 The electromyogram impulse frequency registered during a period of 1/3 second at the end of inspiration bears a linear relationship to the inspiratory muscular work

4 The electromyogram impulse frequency registered within an interval of 1/3 second four seconds after the end of inspiration and with the subject holding his breath with open glottis is directly proportional to the alveolar relaxation pressure

5 During one and the same inspiration the electromyogram impulse number is directly proportional to the pressure impulse This means that during one inspiration the electromyogram impulse frequency registered from the external intercostal muscle bears a linear relationship to the inspiratory muscular force (P_m) The regression coefficient or the slope of this relationship is determined at the beginning of inspiration and varies between different inspirations

6 These linear relationships were also observed during CO stimulation

7 Evidence was obtained of a change of the slope in the electro mechanical relationship during $5 \pm 0.4\%$ CO air stimulation

5 On the basis of the results obtained, it is suggested that the inspiratory centres determine in advance which inspiratory muscles to participate in the inspiratory excursion and which is the strength of contraction of the muscles The electromyogram impulse frequency at the end of inspiration during stable conditions is in a logarithmic relation to the tidal volume The central nervous system thus seems to program each inspiration to reach a predetermined end state

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APPENDIX

TABLE 1

NUMERICAL VALUES OF REGRESSION COEFFICIENTS AND CONSTANTS DERIVED FROM THE EQUATIONS $n = b_n H_t + c_n$, $f_d = b_d W + c_d$ AND $f = b P_{( )} + c$ IN RESPECT OF THE EXPERIMENTAL SUBJECTS. n = number of EMG impulses during inspiration. H = the first time integral of the inspiratory muscular work (cm H_2O × sec × l). f_d = EMG impulse frequency at the end of inspiration (sec^{-1}). W = inspiratory muscular work (cm H_2O × l). f = EMG impulse frequency of the static phase (sec^{-1}). $P_{st( )}$ = alveolar relaxation pressure (cm H_2O)

Experimental subject	Number of inspirations N	Regression coefficient b cm H_2O^{-1} × sec^{-1} × l^{-1}	Constant c	Regression coefficient b_d cm H_2O^{-1} × sec^{-1} × l^{-1}	Constant c_d sec^{-}	Regression coefficient b cm H_2O^{-1} × sec^{-1}	Constant c sec^{-1}	Correlation coefficient r	Value of t	Stat sign $p <$	Modified standard error $\|\delta_y\|$
	19	6 4	5 7					0 65	3 53	0 01	76 7
1	19			5 2	2 9			0 86	6 95	0 001	13 4
	24					13 4	−83 0	0 91	10 99	0 001	24 6
	29	7 3	128 2					0 92	12 50	0 001	49 5
2	20			5 8	60 9			0 91	9 31	0 001	16 3
	32					9 0	7 0	0 90	11 30	0 001	19 8
	37	9 5	124 2					0 80	7 85	0 001	116 2
3	30			8 5	40 0			0 95	16 10	0 001	24 4
	34					20 1	−19 5	0 95	17 21	0 001	2_ 3
	12	9 3	−1 6					0 93	8 00	0 001	49 1
4	12			5 5	21 8			0 88	5 86	0 001	37 9
	25					15 6	−71 2	0 90	9 90	0 001	20 3
	16	4 2	71 2					0 93	9 47	0 001	27 4
5	15			3 0	62 0			0 91	7 91	0 001	23 9
	17					8 8	7 7	0 89	7 56	0 001	23 9

6	16 20 10	8 6	;4 3	3 9	48 5	10 8	−19 2	0 ?0 0 97 0 94	20 26 16 93 11 36	0 001 0 001 0 001	13 8 15 8 17 9
7	41 4? 39	6 7	?1 1	4 0	38 6	10 8	−24 5	0 94 0 90 0 8	17 ? 13 06 7 58	0 001 0 001 0 001	25 4 27 9 22 0
8	19 17 ?0	6 0	4 3	4 ?	7 1	1?5	−70 4	0 80 0 93 0 87	5 50 9 90 7 40	0 001 0 001 0 001	40 2 2? 8 30 8
9	11 10 10	7 ?	78 2	4 6	62 1	11 9	5 9	0 97 0 88 0 88	11 97 5 24 ? 24	0 001 0 001 0 001	27 ? 27 8 30 9
10	40 34 57	5 3	49 7	3 ?	17 9	11 3	−71 2	0 83 0 86 0 96	9 17 9 53 25 43	0 001 0 001 0 001	44 3 20 3 14 5
11	16 12 12	8 6	4 0	3 ?	?0 3	11 8	−31 5	0 94 0 76 0 6?	10 31 3 ?0 2 0	0 001 0 01 0 05	41 3 41 1 45 0
12	9 9 10	? 8	?0 3	3 9	16 3	10 9	− ?7	0 98 0 96 0 96	13 03 9 07 9 70	0 001 0 001 0 001	25 2 20 8 16 2
13	35 37 35	6 1	21 1	4 4	−4 6	13 6	−113 9	0 85 0 77 0 87	9 27 7 14 10 14	0 001 0 001 0 001	68 9 40 1 28 6
14	37 15 2?	11 4	0 2	10 7	−40 0	10 7	−48 8	0 89 0 95 0 93	11 5? 10 07 7 14	0 001 0 001 0 001	92 1 30 2 34 1

TABLE 2

THE EFFECT OF CO_2 STIMULATION ON THE ELECTRICAL AND MECHANICAL PARAMETERS IN THE INTERCOSTAL MUSCLES MENTIONED IN TABLE 1 A = before B = during and C = after (14 ± 2 min) CO_2 stimulation (5.0 ± 0.4% CO_2 air) t_b = value of t test by testing the significance of the difference of two regression coefficients t_c = similar value derived on testing significance of the two constants

Subject	Experimental condition	Number of inspirations N	Regression coefficient b $cmH_2O^{-1} \times sec^{-1} \times l^{-1}$	Constant c	Regression coefficient b $cmH_2O^{-1} \times sec^{-1} \times l^{-1}$	Constant c_d sec^{-1}	Regression coefficient b $cm\ H_2O^{-1} \times sec^{-1}$	Constant c sec^{-1}	Value of t_b	Stat sign $p<$	Value of t_c	Stat sign $p<$	Correlation coefficient r	Value of t	Stat sign $p<$	Modified standard error $\|s_y\|$
1	A	8	9.6	41.9									0.90	8.40	0.001	33.7
		8			6.0	−2.8							0.97	9.77	0.001	19.9
		9					14.2	−67					0.89	4.78	0.01	31.3
	B	8	6.0	75.4					2.06		0.34		0.88	4.64	0.01	34.6
		8			3.6	27.9			3.90	0.01	1.66		0.99	17.19	0.001	12.6
		8					10.3	13.0	1.08		1.49		0.83	4.78	0.01	25.2
	C	9	12.7	−11.4					3.21	0.01	[illegible]14	0.05	0.94	7.23	0.001	33.5
		9			5.8	50.7			1.70		0.84		0.86	4.46	0.01	40.1
		10					15.1	−48	1.56		1.20		0.92	6.64	0.001	23.0
2	A	13	7.1	81.[illegible]									0.86	[illegible]	0.001	44.4
		13			4.2	41.4							0.96	5.59	0.001	26.6
		10					15.1	−74.[illegible]					0.81	3.91	0.01	31.2
	B	14	5.3	106.8					0.58		0.77		0.97	6.11	0.001	31.7
		14			3.5	70.2			0.64		1.12		0.70	4.46	0.001	30.2
		12					13.7	−72.[illegible]	0.26		0.03		0.72	3.28	0.01	38.0
	C	1[illegible]	6.3	6[illegible].1					0.36		1.8[illegible]		0.[illegible]1	6.34	0.001	3[illegible].5
		14			3.2	59.9			0.35		0.45		0.85	5.59	0.001	25.2
		11					13.7	−89.1	0.00		0.21		0.94	8.27	0.001	17.[illegible]

3	A	37 30 34	9 5	124 2	8 5	40 0	20 1	−18 [illegible]					0 80 0 95 0 9	7 89 16 10 17 21	0 001 0 001 0 001	1 07 21 4 [illegible]2 3
	B	8 8 11	5 1	146 2	[illegible]	384	117	−34 1	[illegible] 6 3[illegible] 0 17	0 01 0 001	0 46 [illegible] 0 60	0 01	0 87 0 78 0 95	4 3[illegible] 3 0[illegible] 0 13	0 01 0 00[illegible] 0 001	30 7 18 1 17 1
	C	10 9 8	10 5	38 8	6 5	56 6	20 7	−31 [illegible]	3 0[illegible] 4 14 0 42	0 01 0 001	1 06 [illegible] 0 11		0 32 0 98 0 99	0 64 13 03 17 19	0 001 0 001 0 001	[illegible] 15 3 9 7
4	A	1[illegible] 1[illegible] [illegible]	9 4	−1 0	5 5	21 8	15 6	−[illegible]1 2					0 33 0 58 0 90	8 00 5 90 9 90	0 001 0 001 0 001	43 1 37 0 20 3
	B	9 8 9	7 6	12 6	3 3	13 0	15 7	−84 6	0 83 1 96 0 03		0 27 0 28 0 21		0 85 0 89 0 84	4 27 4 78 4 10	0 01 0 01 0 01	38 1 24 0 39 7
	C	10 10 7	11 7	−6 0	8 9	−40 6	18 4	−103 [illegible]	1 6[illegible] 5 09 0 65	0 001	0 36 2 02 0 29		0 11 0 96 0 97	6 1 9 70 8 9	0 001 0 001 0 001	38 6 20 3 16 2
5	A	16 15 17	4 [illegible]	71 [illegible]	3 9	69 0	8 8	7 7					0 93 0 91 0 89	9 47 7 91 7 56	0 001 0 001 0 001	[illegible]7 4 [illegible]3 9 23 9
	B	10 9 7	3 7	111 6	9 0	73 2	6 1	33 [illegible]	0 9[illegible] 3 3 0 98	0 01	3 18 0 6[illegible] 0 3[illegible]	0 01	0 32 0 92 0 9[illegible]	6 64 6 21 [illegible]	0 001 0 001 0 01	17 3 12 2 [illegible]8 0
	C	12 1[illegible] 11	8 8	77 4	4 0	64 [illegible]	10 7	−19 7	1 75 5 60 1 65	0 001 0 001	1 30 0 63 0 78	0 01	0 38 0 97 0 1[illegible]	15 57 1[illegible] 62 9 13	0 001 0 001 0 001	[illegible] 4 8 2 21 9
6	A	16 20 19	8 6	54 3	3 0	48 5	10 8	−19					0 99 0 97 0 97	26 [illegible]6 16 93 16 45	0 001 0 001 0 001	13 8 1[illegible] 8 17 9
	B	9 7 8	5 4	163 4	4 2	17 2	11 8	− 9 6	[illegible]49 0 39 0 40	0 05	[illegible] 6 1 00 0 16	0 05	0 85 0 93 0 10	4 27 5 66 5 06	0 01 0 01 0 01	66 8 [illegible]6 7 20 6
	C	13 13 10	7 8	32 1	4 1	11 [illegible]	15 1	−[illegible]3 7	1 [illegible]6 0 09 0 92		2 04 0 14 0 91		0 85 0 8[illegible] 0 89	5 35 4 75 5 52	0 001 0 001 0 001	63 5 47 0 34 2

Number of EMG impulses as a function of the pressure impulse produced by inspiratory muscles during one and the same inspiration expressed by equation $n = b_i I + c$ where b_i is the regression coefficient and c constant The tables include the total number of EMG impulses (n) duration of the inspiration (t) tidal volume (V) work of inspiratory muscles (W) the first time integral of inspiratory muscular work (H_t) the coefficient of linear correlation (r) A = before B = during and C after 5 ± 0 4% CO_2 stimulation Each table (3 a b c d e f) relates to different subjects

TABLE 3 a

Experimental condition	Inspiration	Number of points	Regression coefficient	Constant	Number of EMG impulses	Duration of inspiration	Tidal volume	Work of inspiratory muscles	Physical action of inspiratory muscles	Pressure impulse produced by inspiratory muscles	Correlation coefficient
		N	b cm H_2O^{-1} × sec^{-1}	c	n	t sec	V l	W cm H_2O l	H_t cm H_2O × sec × l	I cm H_2O × sec	r
A	1	4	5 7	5 9	75	1 05	0 87	10 5	1	11 6	0 998
	2	5	7 0	7 0	100	1 31	0 77	7 8	5 5	13 3	1 000
	3	5	7 9	8 4	123	1 15	1 28	17 9	9 1	14 7	0 998
	4	6	6 7	14 5	261	1 35	2 86	80 8	5 0	36 4	1 000
	5	5	8 7	−0 1	255	1 17	2 32	62 3	34 7	29 2	1 000
	6	6	7 2	2 7	333	1 50	3 37	115 3	75 6	40 0	0 999
B	7	5	7 6	29 2	158	1 15	1 84	31 4	13 7	18 2	0 997
	9	6	7 5	15 9	208	1 35	2 12	43 2	23 3	25 5	1 000
	9	5	7 5	22 0	234	1 30	2 54	56 4	31 8	27 7	0 999
	10	5	7 7	18 7	205	1 15	2 45	55 2	28 8	24 5	0 999
	11	6	7 9	22 3	307	1 55	2 98	72 5	45 2	36 7	1 000
	12	8	6 8	29 2	472	2 00	4 06	138 0	113 3	65 0	0 999
C	13	5	7 1	10 2	76	1 10	0 72	6 4	3 0	9 2	0 999
	14	6	7 8	13 5	85	1 30	0 74	5 8	2 9	9 4	0 990
	15	5	8 6	10 2	137	1 30	1 12	13 1	7 0	14 6	0 999
	16	7	10 6	9 3	200	1 55	1 63	25 6	17 6	23 5	1 000
	17	6	11 2	10 4	301	1 60	2 06	37 2	22 7	25 9	1 000
	18	7	12 4	7 4	406	1 70	2 47	47 0	34 7	32 4	1 000

TABLL 3 b

Experimental condition	Inspiration	Number of points N	Regression coefficient b cm H_2O ^{1} × sec^{-1}	Constant c	Number of EMG impulses n	Duration of inspiration t sec	Tidal volume V 1	Work of inspiratory muscles W cm H_2O × 1	Physical action of inspiratory muscles H_t cm H_2O × sec × 1	Pressure impulse produced by inspiratory muscles I cm H_2O × sec	Correlation coefficient r
A	1	8	3 4	3 3	45	1 8[illegible]	0 89	6 3	4 5	11 7	0 997
	2	9	6 0	17 2	141	2 [illegible]5	1 48	15 1	14 4	21 6	0 990
	3	9	8 6	[illegible]0 4	[illegible]18	2 30	1 [illegible]3	19 2	17 7	23 [illegible]	0 999
	4	8	11 0	10 8	352	2 00	3 21	50 0	39 0	30 9	1 000
	5	8	7 3	16 9	198	2 00	2 28	3[illegible] 7	2[illegible] 3	25 2	0 997
	6	10	10 5	19	[illegible]07	2 40	4 08	89 1	79 9	45 4	0 998
B	7	7	3 6	10 1	74	1 6[illegible]	1 37	16 4	11 1	18 5	0 993
	8	8	5 2	[illegible]08		1 8[illegible]	[illegible] 33	48 8	45 7	36 4	0 999
	9	12	[illegible] 5	14 0	337	2 90	2 84	56 5	8[illegible] 4	[illegible]7 0	0 999
	10	7	[illegible] 4	16 6	13[illegible]	1 65	1 [illegible]4	30 4	20 6	23 0	0 996
	11	8	7 6	23 9	307	1 90	3 04	64 1	46 9	36 3	0 998
	12	10	10 3	[illegible]7 5	6[illegible]3	2 45	3 [illegible]8	105 2	9[illegible] 0	55 0	0 995
C	13	6	7 0	7 8	6	1 [illegible]0	0 77	[illegible] 0	3 5	10 1	0 99[illegible]
	14	9	[illegible] 6	17 3	186	2 10	1 34	14 7	14 9	[illegible]2 4	0 997
	15	10	11 1	[illegible]8 2	400	2 50	2 24	34 2	35 2	34 1	0 9[illegible]8
	16	8	8 2	19 0	199	1 [illegible]5	1 78	24 7	18 4	22 0	0 997
	17	7	8 8	16 7	[illegible]37	1 75	2 1[illegible]	34 4	23 7	24 7	0 997
	18	10	10 3	12 1	481	2 30	2 85	61 3	6[illegible] 3	44 7	0 999

TABLE 3c

Experimental condition	Inspiration	Number of points N	Regression coefficient b cm H_2O l^{-1} × sec^{-1}	Constant c	Number of EMG impulses n	Duration of inspiration t sec	Tidal volume V l	Work of inspiratory muscles W cm H_2O × l	Physical action of inspiratory muscle H_i cm H_2O × l × sec	Pressure impulse produced by inspiratory muscles I cm H_2O sec	Correlation coefficient r
A	1	6	4.5	−12.6	73	1.60	1.35	1[illegible].5	12.4	15.5	0.997
	2	7	10.3	0.6	[illegible]00	1.70	1.52	17.7	13.4	18.8	0.998
	3	9	11.3	7.2	302	2.00	1.65	21.0	24.5	25.9	0.999
	4	6	8.1	2.9	171	1.50	2.00	28.1	20.0	20.[illegible]	0.998
	5	8	10.6	−27.5	415	1.95	3.08	67.8	73.0	41.[illegible]	0.999
	6	10	11.2	1.6	604	2.45	3.37	85.0	118.1	59.9	0.999
B	7	7	4.9	12.8	103	1.70	1.00	13.4	10.4	19.2	0.992
	8	6	3.3	14.0	79	1.55	1.60	22.4	17.0	20.1	0.996
	9	9	7.9	22.1	271	2.10	1.88	29.4	31.0	30.8	0.998
	10	8	8.9	32.9	312	1.90	2.13	38.4	36.5	31.3	0.999
	11	8	9.0	0.1	353	1.90	2.90	67.9	57.1	38.0	0.990
	12	7	7.5	23.3	406	1.70	4.00	129.8	94.1	50.4	1.000
C	13	6	4.8	8.5	72	1.45	1.00	9.7	7.2	13.9	0.996
	14	7	4.8	10.6	8[illegible]	1.60	1.02	10.4	8.1	15.1	0.996
	15	7	8.5	4.5	185	1.60	1.50	21.1	17.5	20.8	0.999
	16	7	12.3	12.4	306	1.70	1.50	22.3	19.5	23.4	1.000
	17	6	12.1	−3.5	295	1.55	1.75	29.1	24.4	24.1	0.999
	18	11	11.4	−29.1	555	2.60	2.45	48.3	75.5	49.2	0.996

TABLE 3 d

Experimental condition	Inspiration	Number of points N	Regression coefficient b cm H_2O^{-1} × sec^{-1}	Constant c_i	Number of EMG impulses n	Duration of inspiration t sec	Tidal volume V l	Work of inspiratory muscles W cm H_2O × l	Physical action of inspiratory muscles H_t cm H_2O × sec × l	Pressure impulse produced by inspiratory muscles I cm H_2O × sec	Correlation coefficient r
A	1	4	11 2	20 9	89	1 10	0 87	5 3	2 7	6 2	0 997
	2	5	12 1	18 4	158	1 40	1 03	8 6	7 7	11 5	1 000
	3	6	15 1	0 6	[illegible]09	1 60	1 2[illegible]	9 0	8 0	12 4	0 998
	4	0	17 5	18 4	4[illegible]0	2 30	1 3[illegible]	13 0	20 4	22 4	0 999
	5	7	21 4	15 8	451	1 90	1 74	18 8	20 4	20 0	0 999
	6	8	18 1	11 4	484	2 10	2 63	33 7	33 8	26 1	1 000
B	7	4	10 3	8 4	104	1 10	1 64	14 0	6 6	9 3	1 000
	8	5	10 8	13 3	114	2 10	1 [illegible]	9 2	9 2	15 4	1 000
	9	8	9 4	6 0	161	2 10	1 25	9 7	9 8	16 3	1 000
	10	6	9 9	5 3	169	1 40	2 24	26 3	18 1	17 1	0 999
	11	9	10 5	18 8	307	2 30	2 02	23 8	34 4	27 5	0 999
	12	13	10 2	16 3	37	3 [illegible]0	2 28	32 3	[illegible]2	49 7	0 999
C	13	6	1[illegible] 4	−3 7	1[illegible]2	1 40	1 06	6 0	3 9	8 1	0 [illegible]38
	14	5	1[illegible] 0	1[illegible] 0	109	1 [illegible]0	1 28	8 8	4 0	8 3	0 997
	15	9	14 6	15 6	293	2 30	1 49	11 4	1[illegible] 3	19 1	0 999
	16	7	19 6	19 3	[illegible]87	1 [illegible]0	1 98	17 0	1[illegible] 0	13 9	0 998
	17	7	17 1	13 7	301	1 [illegible]0	2 12	22 0	17 [illegible]	16 7	0 999
	18	8	21 4	18 1	471	2 00	2 84	31 8	25 4	21 4	1 000

TABLE 3 e

Experimental condition	Inspiration	Number of points	Regression coefficient	Constant	Number of EMG impulses	Duration of inspiration	Tidal volume	Work of inspiratory muscles	Physical action of inspiratory muscles	Pressure impulse produced by inspiratory muscles	Correlation coefficient
		N	b cm H_2O^{-1} × sec^{-1}	c_i	n	t sec	I l	W cm H_2O × l	H_i cm H_2O × sec × l	I cm H_2O × sec	r
A	1	6	3 5	4 5	42	1 50	0 58	3 9	3 1	11 1	0 998
	2	8	4 2	12 5	86	2 03	0 88	8 0	7 7	18 4	0 991
	3	7	5 6	10 2	121	1 81	1 14	12 4	11 2	20 2	0 996
	4	9	7 1	21 2	199	2 19	1 34	15 8	16 0	25 7	0 997
	5	8	8 4	16 8	265	2 10	1 99	29 8	26 5	29 6	0 999
	6	13	8 9	−6 4	737	3 15	4 43	116 2	152 3	9 9	0 998
B	7	5	6 0	−0 3	163	1 25	1 14	26 0	13 4	27 7	0 999
	8	5	6 1	0 1	165	1 30	1 31	28 8	21 1	27 6	0 999
	9	6	8 5	−1 5	345	1 50	1 78	50 4	37 3	40 6	1 000
	10	7	8 8	18 7	483	1 65	1 94	65 6	56 4	51 7	0 999
	11	7	8 4	0 6	488	1 70	1 96	65 1	70 0	54 5	0 997
	12	8	7 6	−0 2	515	2 00	2 69	94 6	93 1	60 5	1 000
C	13	9	5 0	18 2	125	2 30	0 91	8 5	10 2	22 4	0 993
	14	6	5 7	18 1	120	1 50	1 06	13 0	10 0	17 7	0 993
	15	9	7 2	22 4	285	2 31	1 92	32 2	35 9	36 3	0 997
	16	7	9 5	25 7	344	1 82	2 04	30 1	36 2	33 2	0 998
	17	9	6 8	16 8	277	2 25	2 05	37 5	43 0	37 0	0 999
	18	8	8 4	11 5	468	2 04	3 20	96 6	90 5	52 8	0 999

TABLE 3f

Experimental condition	Inspiration	Number of points	Regression coefficient	Constant	Number of EMG impulses	Duration of inspiration	Tidal volume	Work of inspiratory muscles	Physical action of inspiratory muscles	Pressure impulse produced by inspiratory muscles	Correlation coefficient
		N	b cm H_2O^{-1} × sec^{-1}	c	n	t sec	V l	W cm H_2O × l	H_t cm H_2O × sec × l	I cm H_2O × sec	r
A	1	6	11 9	2 6	150	1 50	1 16	10 2	6 8	11 6	0 995
	2	7	14 7	20 1	253	1 60	1 95	21 3	12 9	15 6	0 994
	3	8	13 6	9 0	373	1 80	2 50	37 9	31 9	25 8	0 999
	4	9	13 1	35 0	584	2 25	3 88	76 6	68 8	41 6	0 990
	5	10	11	39 8	703	2 40	4 75	120 5	141 9	58 8	1 000
	6	6	12 1	3 5	174	1 30	1 79	21 2	10 8	14 0	1 000
B	7	8	5 7	20 6	189	2 00	2 07	32 9	30 4	31 0	0 994
	8	7	10 4	24 2	399	1 70	2 18	64 8	53 2	34 6	0 998
	9	8	10 8	32 1	433	1 85	3 30	72 4	59 8	37 2	0 991
	10	8	7 7	24 9	514	2 00	4 95	166 6	151 1	64 0	1 000
	11	6	7 0	17 6	107	1 40	1 04	10 3	7 5	12 8	0 993
	12	7	10 8	37 3	402	1 60	3 08	0 6	0 2	33 6	[illegible]
C	13	6	13 5	37 7	215	1 50	1 75	16 3	[illegible]	14 3	[illegible]
	14	6	13 6	30 8	302	1 50	2 61	36 0	[illegible] 1	20 2	0 998
	15	7	19 3	19 7	16	1 [illegible] 0	1 [illegible] 4	11 9	10 3	15 7	0 998
	16	7	18 1	39 3	40	1 60	2 [illegible]	[illegible]	[illegible]	[illegible] 0 3	0 [illegible] 6
	17	8	13 0	30 8	4 [illegible]	1 30	[illegible]			[illegible]	1 000
	18	8	10 9	18 4	[illegible] 7	2 00	[illegible]	[illegible]		[illegible]	0 999

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 297

POSTNATAL CHANGES IN CUTANEOUS REFLEXES AND IN THE DISCHARGE PATTERN OF CUTANEOUS AND ARTICULAR SENSE ORGANS

A MORPHOLOGICAL AND PHYSIOLOGICAL STUDY IN THE CAT

BY

JAN EKHOLM

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CONTENTS

CHAPTER I

GENERAL INTRODUCTION

Following the neuro histological studies of the embryonic central nervous system by *His* (1889) and *Ramon y Cajal* (1890 a b) which led to the formulation of the neurone theory the physiology of developmental mechanisms attracted great interest During the first four decades of this century several extensive investigations on this subject were published some concerning behavioral aspects (e g *Graham Brown* 1914, 1915 *Coghill* 1914 1916 1926a b 1930, *Weed* 1917, *Langworthy* 1925, 1926 1929, *Minkowski* 1921 a, b 1923 *Windle* 1929 1934 1940, *Angulo y González* 1932 1937 *Coronios* 1933 *Carmichael* 1933 1934 a b *Hooker* 1937) Since the contemporary physiological techniques were rather crude both in comparison with those now available and compared with the morphological techniques of that time no detailed analyses of the mechanisms behind developmental changes were possible

The physiological studies consisted chiefly of determinations of when and where in the organism different reflexes appeared or disappeared during the process of development These findings are obviously indispensable for studies of developmental mechanisms but give no information about the developmental changes in different parts of a reflex With modern electro physiological techniques however, it is possible to study separately both the afferent and the efferent links of a reflex in detail and thereby uncover the links in a chain of developmental events

In 1960 *Skoglund* published a series of important papers regarding the postnatal developmental of postural reflexes (for review see *Skoglund* 1966) He found that the decerebrate rigidity and tonic stretch reflexes are absent in the hindlimbs of newborn kittens, whereas motor activity is easily elicited from the skin demonstrating that the efferent side of the stretch reflex path is functioning He further showed that the muscle sense organs only give phasic discharges of low frequency at birth and that tonic discharges do not appear until the conduction velocity of the afferent fibres has reached a certain level These findings explain the absence of tonic stretch reflexes in newborn kittens especially since it was also found that the muscle spindles lack gamma innervation for some time after birth which provides the explanation for the absence of decerebrate rigidity (*Granit* 1955) The

effectiveness of skin stimuli as against activation of muscle receptors in evoking motor responses in the newborn kitten poses several questions, two of which constitute the main theme of this thesis

1) Are the cutaneous sense organs functionally more mature than the muscle receptors which would explain their greater influence on motor activity?

2) Are the cutaneous sense organs as immature functionally as the muscle receptors and do their properties change with the same relationship between function and fibre size as in muscle sense organs? If so is their greater effectiveness in evoking motor activity a consequence of the absence or immaturity of supraspinal regulation and segmental postural reflexes?

In order to throw light on these questions a study has been made of functional changes of the cutaneous sense organs the morphological development of such nerve fibres and alterations in the patterns of spinal reflexes elicited from the skin in cats of different stages of maturation from newborn to adult

CHAPTER II

ANATOMY AND NOMENCLATURE

For the convenience of the reader a description of the gross anatomy of the relevant cutaneous nerves and the nomenclature used is presented Most of the information is available in the literature (*Stowell* 1891 *Horsburgh* and *Heath* 1950 *Reighard* and *Jennings* 1961 *Harrison* 1962) but some new observations have been added Most of these were made in connection with the preparation of the animals for experiments or biopsy but some animals were also dissected systematically When the innervation fields of the skin nerves were determined the nervous activity set up by mechanical stimulation of the skin was recorded from the proximal parts of the nerves (for method of recording see Chapter III) The mechanical stimulation was performed by means of a sable brush (Chapter IV)

The nomenclature used has been adapted from the textbooks mentioned above and generally modified according to the Nomina Anatomica (1964)

The following cutaneous nerves have been of interest in the present study (the abbreviations used are given in brackets)

N cutaneus femoris lat (n cut fem lat) whose branches are distributed over the lateral aspect of the thigh extending distally to the knee joint region enters the thigh by penetrating the abdominal wall near spina iliaca ant sup (Fig 1) It accompanies a ilio lumbalis and is often divided into two main trunks

N saphenus is a branch of n femoralis It passes initially along a femoralis (Fig 1) and more distally on the medial side of the knee joint and along the lower limb Its end branches extend to the ankle joint region and to the dorsum of the foot

N cutaneus femoris post (n cut fem post) is usually found between tuber ischii and trochanter major (Fig 1) where the nerve emerges through the muscles It gives off branches in medial and lateral directions The main branch continues along the popliteal fossa It usually extends distally on the posterior part of the calf accompanying v saphena parva (cf *Reighard* and *Jennings* 1961)

N suralis usually originates from n tibialis or from n ischiadicus (sciatic nerve) proximal to its division into n tibialis and n peroneus communis N suralis pierces the adipose tissue in the popliteal fossa and lies usually on

the posterior surface of m gastrocnemius (Fig 1) The main branch passes to the heel where it gives off twigs for the heel region and the latero plantar aspect of the foot Some twigs are often crossed by twigs of n cut fem post, the latter pass medially more superficially than the branches of n suralis A lateral branch sometimes originating from n suralis and sometimes directly from n tibialis or n ischiadicus often gives off branches in the lateral part of the knee joint region and the lower limb

Nn plantaris med et lat are branches of n tibialis and emerge from behind the medial malleolus The nerves give off branches to the plantar surface of the foot and toes

N peroneus superficialis (n per superf) becomes superficial near the ankle joint region on the anterior side of the lower limb It supplies the ankle region the dorsum of the foot and the toes

N peroneus profundus (n per prof) gives off some cutaneous branches on the dorsum of the tarsus and supplies the lateral digits

Variations observed in the present study

1) Anastomoses were often noticed between n suralis and n cut fem post between n suralis and n per superf between n cut fem post and n per superf and between n per superf and n saphenus It appeared as if fibres of the distal branches of the different nerves came together and then ran in a common strand

2) In three out of some 150 preparations n suralis was completely embedded in muscular tissue for about 1 cm of its length in the proximal part of m gastrocnemius In none of these cases was the nerve embedded in the gastrocnemius muscle on the opposite side

3) N cut fem post varied in its length According to *Reighard* and *Jennings* (1961) it may be traced as far distal as the popliteal space In the present study however it was found that in most cases this nerve extended down to and sometimes beyond the heel region There seemed to be a relationship between the magnitude of n suralis and n cut fem post When n suralis was thin and extended only a short distance distally n cut fem post was large and gave off branches far distally in the area that n suralis normally supplied In 2 cases out of some 150 the main branch (medial) of n suralis could not be found but n cut fem post was large and supplied the area The opposite situation was also seen when n cut fem post was small and n suralis [illegible] ch variations were unilateral the other side hav[illegible] com[illegible] of distribution

Innervation fields of the cut[illegible] es in [illegible]

The skin area supplied by [illegible] ous [illegible] blished exactly by dissection The

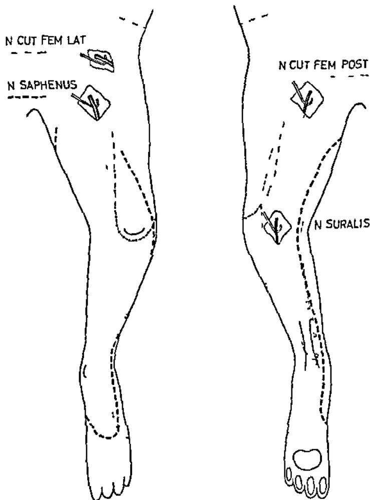


FIG 1 Diagram showing the innervation fields and proximal main trunks of cutaneous nerves on the anterior and posterior aspects of the hindlimb

cutaneous nerves in the cat was performed by means of an electrophysiological technique

Fig 1 shows the innervation fields of different cutaneous nerves on the anterior and posterior aspects of the hindlimb in a cat *N saphenus* supplied a large area on the antero-medial aspect of the thigh lower limb and proximal part of the dorsum of the foot In one case this nerve consisted of two naturally separated fasciculi These two fasciculi were investigated separately and it was found that there was very little overlapping between the two innervation fields One of the natural fasciculi

was then artifically divided into two new fasciculi of approximately equal thickness The innervation fields of the two new artificial fasciculi were tested separately and it was found that these two fields were almost identical *N cut fem lat* supplied an area on the lateral aspect of the thigh This area extended far enough anteriorly and posteriorly to overlap somewhat on the areas of the n saphenus and n cut fem post On the posterior surface of the hindlimb the area supplied by *n cut fem post* can be seen This area is situated on the posterior surface of the thigh and lower limb *N suralis* usually supplied the postero lateral part of the lower limb the heel region and the lateral margin of the foot The distal border of the field varied, sometimes it extended to the middle of the foot sometimes to the lateral toe Some overlap between different areas subserved by the nerve trunks was observed but this was not as great as that of the dermatomes (see Chapter IV) The innervation fields of the cutaneous nerves studied were not exactly bisymmetrical

The following terms will be used

buttock	=	the caudal region ventro lateral to the base of the tail
thigh	=	the part of the hindlimb between the hip and knee joints
lower limb (leg)	=	the part of the hindlimb between the knee and ankle joints
heel	=	the region at the posterior end of the calcaneum
foot pad	=	plantar cushion = the central pad of the sole of the foot
toe pads	=	the peripheral pads on the toes
rootlet	=	naturally occurring subdivisions of spinal roots at the entrance into the spinal cord
afferent unit	=	peripheral afferent unit (sensory unit) = afferent axon its receptor terminals or endings and any associated non nervous structural elements (*Iggo* 1966 p 240)

CHAPTER III

SEGMENTAL ORIGIN OF CUTANEOUS NERVES

Introduction and literature

Stowell (1882 1886, 1887 1891) dissected the peripheral nervous system in the cat In 1891 he described the lumbo sacral plexus and its peripheral nerves With his macro dissection method it was only possible to study the segmental origin of such nerves that originated from the plexus separately The segmental origin of branches to larger nerves (e g nn suralis and saphenus) could not be traced by this method The segmental origin of n cut fem lat was reported to be L5—L6 Elsewhere in the text however *Stowell* reported The exposure of the myelin in the arch of the caudal thoracic vertebra (thirteenth) exposes the ectal origin of the first lumbar nerve This probably means that the nerve that he denominated L1 is the same one as is usually denominated Th 13 An analogous situation seems to exist with *Stowell s* description of the first sacral nerve the largest of the spinal nerves it traverses the long groove in the seventh lumbar vertebra and finds its exit through the foramen mesad of the crista ilii The root described in this way must be the one usually denominated L7

According to *Stowell* (after correction of the root number) n cut fem lat should originate from L4—L5 This is consistent with some textbooks of cat anatomy (*Reighard* and *Jennings* 1961 *Harrison* 1962)

N cut fem post has been reported to originate from S2—S3 (*Reighard* and *Jennings* 1961) and from S1—S3 (*Harrison* 1962)

The segmental origin of cutaneous nerves has been investigated also by means of electrophysiological techniques *Widen* 1951 (cited by *Hagbarth* 1952) showed that n suralis and n cut fem post could be mainly referred to dorsal roots S1 and L7 while n saphenus could be mainly referred to L6 and L5

In the present work recordings had to be made from dorsal root fibres connected to cutaneous sense organs (Chapter VII) It then had to be determined where the cutaneous afferents of a certain nerve or from a certain region (Chapter II) reached the spinal cord Furthermore for the study of cutaneous reflexes (Chapter VIII) knowledge of the segmental

origin of the stimulated nerves was of value. Since the results presented in the literature concerning these questions are incomplete and somewhat controversial, the present study was performed with the two following questions in mind: 1) Into what dorsal roots do fibres from a certain cutaneous nerve enter? 2) Are the subdivisions of the dorsal root, i.e. the dorsal rootlets, organized in such a way that fibres of a certain nerve can be constantly found in one or more certain rootlets?

Material and Methods

Seven adult cats were used and denominated by consecutive numbers. They were anaesthetized with pentobarbital 40 mg/kg body weight (b.w.) intraperitoneally (i.p.). A lumbo sacral laminectomy was performed. The dura was divided and the dorsal roots L3—S3 were cut close to the spinal cord and immersed in liquid paraffin. In the hindlimb the cutaneous nerves were dissected free and covered with liquid paraffin. The animals were mounted on a conventional experiment stand. The rectal temperature was checked and maintained near to 37°C by means of adjustable radiant heat. In cats Nos. 1, 2 and 3 the rootlets were placed on Ag—AgCl hooks and stimulated and the responses were recorded from the peripheral nerves. The recording electrode (Ag wire) on the peripheral nerve was always placed proximal to the branches of the nerve. A reference electrode was inserted into inactive tissue. In cats Nos. 4, 5 and 6 the peripheral nerves were stimulated and the recording was made from the rootlets. The two methods gave essentially similar results.

The stimulation was performed by square wave pulses of 0.3 msec duration. The stimulus strength was supramaximal. The potentials were recorded between the active electrode on which the nerve was placed and the reference electrode inserted into inactive tissue through a cathode follower input to an amplifier, displayed on a cathode ray tube and photographed on moving bromide paper. Time was indicated by a sine wave generator on a second beam.

Results

The segmental origins of n. suralis, n. cut. fem. post., n. saphenus and n. cut. fem. lat. are schematically illustrated in Figs. 2—3. Naturally occurring rootlets that were found to have connections with the cutaneous nerve studied are indicated by the black areas in the graphs. The length of each

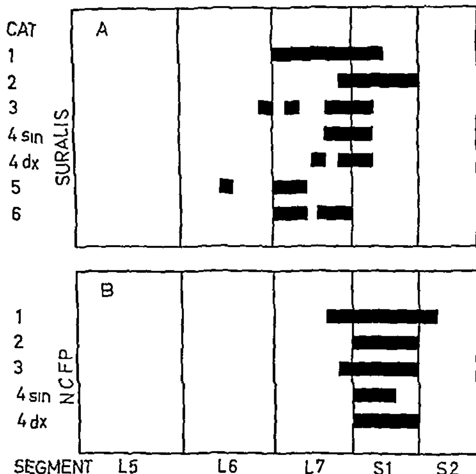


FIG 2 Diagram showing the segmental origin of n suralis (A) and n cut fem post (B) The black areas indicate rootlets found to have connections with the cutaneous nerve

segment in the graphs is roughly proportional to the root entrance zone (*Thomas* and *Combs* 1962)

The segmental origin of n suralis in 6 cats is shown in Fig 2A in one cat both sides were studied (No 4) It can be seen that the fibres of the sural nerves studied originated from the L6 L7 and S1 dorsal roots In each individual animal the origin usually comprised only two segments L7 and S1 in four cases and L6 and L7 in one case Such variations are probably due to the type of pre- and postfixation of the cats In one cat however L6 L7 and S1 were involved and in one case only L7 It can

also be seen in Fig 2A that some fibres of n suralis originated from L7 in all cases Fibres of n suralis did not originate from consecutive rootlets in cats No 3, 4 5 and 6 In cat No 2, for instance there were three rootlets between the two portions of the segmental origin In cat No 3 the origin was divided into three portions In cat No 1 the number of rootlets of each dorsal root studied was less than usual, each rootlet was however thicker than usual In this case no interruption of the origin was observed These findings probably depend not only upon the distribution of the fibres but also upon the thickness of the naturally occurring rootlets from which the recordings were made

In the most caudal rootlets of L7 fibres of n suralis were found in 6 cases out of 7 This finding that fibres can nearly always be found in the most caudal part of L7 (and rostral part of S1) was utilized and confirmed in the work presented in chapter VII The results obtained in cat No 4 showed that the segmental origin of n suralis was not equal on the two sides, which may be a common situation

Fig 2B shows that n cut fem post originated from L7 S1 and S2 In 3 cases out of 5 only S1 was involved Fibres of this nerve were found in

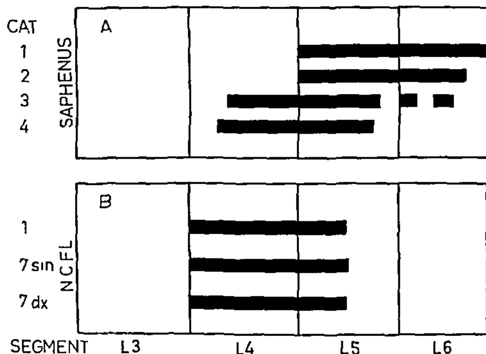


Fig 3 Same as Fig 2 N saphenus (A) and n cut fem lat (B)

the rostral part of S1 in all cases studied The origin of the nerve in cat No 4 was not bisymmetrical

Fibres of n saphenus (Fig 3A) were traced to the dorsal roots L4 L5 and L6 In two cases L5 and L6 were involved in one L4 and L5 and in one L4 L5 and L6 Saphenous fibres were found in the rostral part of L5 in all cases studied

Fig 3B shows the results obtained in the experiments on n cut fem lat In all three cases the fibres of this nerve were found in L4 and the rostral part of L5 (in one case L3 was not tested)

Discussion

When using a method such as this for tracing nerve fibres one has to consider the possible errors involved The ratio between active and inactive fibres at the recording electrode is of importance If the ratio is small the activity of a few fibres might be completely shunted By using recording and stimulation at both ends of the fibres an attempt has been made to minimize such errors

The segmental origins of n cut fem lat n suralis and n saphenus found in the present study are essentially consistent with the reports in the literature

The segmental origin of n cut fem post however was not quite in accordance with those reported in the anatomical textbooks (S2—S3 *Reighard* and *Jennings* 1961 and S1—S3 *Harrison* 1962) This nerve was found to originate mainly from S1 and the caudal part of L7 and only in one case from the rostral part of S2 Fibres of this nerve were never found in the dorsal root S3 The findings are essentially in accordance with those of *Widen* (cited by *Hagbarth* 1952) Thus in contrast to other reports *Widen's* finding was confirmed However some features of the segmental origin of cutaneous nerve fibres ought to be stressed

1 The composition of the segmental origin varies considerably between the specimens 2 Fibres of one cutaneous nerve do not always enter consecutive rootlets 3 On the other hand some fibres of a particular nerve often enter a certain part of a root or a certain rootlet

Summary

1) The segmental origin of different hindlimb cutaneous nerves was studied by electrical stimulation of nerves or rootlets and recording from rootlets or nerves in cats

2) N suralis originated from L6 L7 and Sl but in each animal usually only from two of these segments Fibres of the sural nerve did not enter consecutive rootlets However sural fibres were nearly always found in the caudal rootlet of L7 (and rostral Sl)

3) N cut fem post originated from L7 S1 and S2 but usually from S1 Fibres of this nerve were always found in the rostral part of S1

4) N saphenus originated from L4 L5 and L6 saphenous fibres were always found in the rostral part of L5

5) N cut fem lat originated from L4 and L5

CHAPTER IV

ON THE DISTRIBUTION OF HINDLIMB DERMATOMES

Introduction

The distribution of the dermatomes in the cat was investigated by *Klessens* (1914) He used a strychnine isolation technique hyperreflexive zones (which corresponded to the dermatomes) were mapped out by touching the skin after the application of strychnine to the dorsal root under study The adjacent dorsal roots were sectioned *Kuhn* (1953) and *Hekmatpanah* (1961) used an electrophysiological technique for defining the tactile dermatomes They initiated afferent impulses by mechanical stimulation of the skin and recorded the responses from the sectioned dorsal rootlets The muscle nerves were not sectioned *Kuhn* investigated the segments from L4 to S4 in the cat and *Hekmatpanah* investigated the segments from C1 to L4, also in the cat By recordings from dorsal root afferents *Pubols* et al (1965) made observations of the forelimb dermatomes in the cat

The results of *Klessens* (1914) and *Kuhn* (1953) are not quite in accordance *Kuhn* found that the S1 dermatome innervated only the buttocks and the base of the tail whereas *Klessens* found that it extended more distally The observations of *Kuhn* are those usually cited in the literature *(Celesia* 1963)

When studying the cutaneous reflexes (Chapter VIII) skin stimuli were applied to different regions of the hindlimb and it was therefore important to know which segment supplied the region under study hence the distribution of some hindlimb dermatomes was reinvestigated here

Material and Methods

Cats and kittens were anaesthetized with pentobarbital 40 mg/kg b w i p Since the material was obtained from the same animals that were used for the investigation of the cutaneous sense organs (Chapter VII) it comprised about 100 cats and kittens A lumbo sacral laminectomy was performed and the dorsal roots L6—S1 were exposed and sectioned near the spinal cord (for details see Chapter III) Nn tibialis and peroneus comunis

were cut in order to avoid interference from muscle sense organs This meant that the cutaneous receptive areas of the nn plantares and nn per superf and prof were not included in the dermatomes mapped The configuration presented of the distal ends of the dermatomes on the foot is thus not anatomically correct the true dermatomes may be somewhat larger The proximal parts of the dermatomes near the trunk were not completely investigated

Nerve fibres that were connected to cutaneous sense organs were isolated in dorsal roots by dissecting free thin filaments under the microscope Recording from single dorsal root filaments is described in more detail in Chapter VII The recording equipment was the same as described in Chapter III Stimulation of the skin was usually performed by means of a sable brush and the localization of the receptive fields of the single mechanosensitive units was determined The localization was indicated on drawings

In 4 cats the size and localization of the S1 dermatomes were determined by recordings from the rootlets The muscle nerves were cut (see above) and the borders of the dermatomes were mapped by means of mechanical stimulation of the skin with a sable brush

Results

Figs 4A and 5A C show the skin areas within which receptive fields of mechano sensitive units of the dorsal roots L6, L7 and S1 were localized The observations were made in several different cats and the areas attributed to the different segments represent a sum of these observations The variation between the specimens is thus included As can be seen in Fig 4A the S1 dermatome was situated on the posterior aspects of the thigh calf and foot and extended to the distal part of the foot Sometimes units were also found down to the digits Fig 5A shows the L7 dermatome It had a similar localization but was shifted somewhat laterally as compared with S1 The L6 dermatome (Fig 5B C) was incompletely mapped but it can be seen that part of the dermatome covered the anterior and medial aspects of the lower leg and that this dermatome also extended to the digits

Fig 4B shows the S1 dermatome in one of the cats in which it was mapped by recording from dorsal rootlets The dermatome extended distally to the middle of the plantar surface of the foot Except for the foot the area is essentially in congruence with that obtained by the single unit method (Fig 4A) The dermatomes obtained by recordings from rootlets in the rest of the cats were essentially similar to that illustrated in Fig 4B

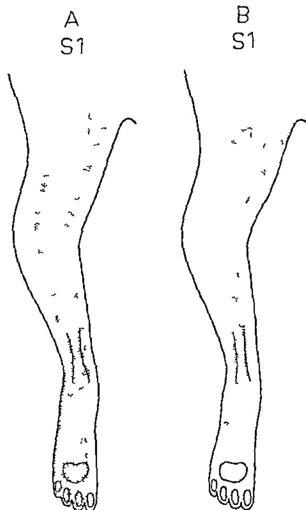


FIG 4 Diagram showing the posterior aspect of the hindlimb with the S1 dermatome (dotted area) delineated by recordings from single units in the dorsal root (A) and from dorsal rootlets (B) N tibialis and n peroneus comunis were sectioned

Discussion

Recordings from rootlets were made in the present work and also in *Kuhn s* study The preparations used however, were not quite comparable since in the present investigation the muscle nerves were sectioned This was considered necessary in view of the fact that the muscle spindles are known to have a very low threshold to mechanical displacements On the other hand this means that some cutaneous branches were also cut which should cause some dermatomes to appear smaller than they actually are With the procedure used here this applies especially to the area on

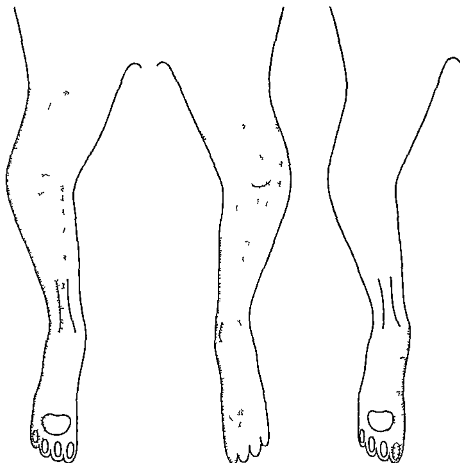


FIG 5 Same as Fig 4(A) The L7 dermatome on the posterior aspect of the hindlimb (A) the L6 dermatome on the anterior (B) and posterior (C) aspects of the limb

the foot By recording from rootlets, the activity in some few fibres may be shunted if the ratio of active to inactive fibres is small Since the same recording method was used in the present work as in *Kuhn s* study this cannot explain the difference in the results In addition single unit recording was used in the present investigation By this method the above mentioned error is excluded On the other hand findings from many animals have to be combined which due to the variation between specimens should tend to make the dermatomes larger than the actual one in each individual animal

The results obtained by mapping the L6 and L7 dermatomes were essen

tially the same as those of *Klessens* (1914) As compared with the L6 and L7 dermatomes found by *Kuhn* (1953) those found in the present investigation were somewhat narrower This difference might be due to the absence of influences from muscle organs The results obtained for the S1 dermatome however disagree with *Kuhn s* description but in the opposite direction to the discrepancies regarding L6 and L7 in a material of 4 animals *Kuhn* showed that the tactile S1 dermatome innervates only the skin of the buttocks and the base of the tail In the present investigation however, cutaneous units of the S1 dorsal root were also found regularly distally to the knee i e on the calf heel and proximal part of the foot and sometimes also in the distal part of the foot This finding is essentially in agreement with the early work of *Klessens* (1914) The distribution of the S1 dermatome was also investigated by *Hagbarth* (1952) who studied the excitatory and inhibitory effect of skin pinching on the activity recorded from the nerve to the ankle extensors before and after sectioning the S1 dorsal root He found that the region over the heel and the middle part of the plantar region were innervated by S1 (p 43)

In this connection *Celesia s* (1963) investigation of the segmental organization of the cortical afferent areas in the cat is of interest On stimulating the S1 dorsal root he found that potentials were recorded not only on the medial aspect of the hemisphere where the buttocks and tail are represented but also on the dorsal aspects of the hemisphere in the hindlimb area Assuming as claimed by *Kuhn* that S1 does not innervate the skin of the hindlimb he suggests that the latter response in the hindlimb area could have resulted from stimulation of fibres originating from the intrinsic muscle of the foot In the light of the present investigation and those of *Klessens* (1914) and *Hagbarth* (1952) however the appearance of responses in the cortical hindlimb area on stimulation of the S1 dorsal root certainly need not be explained by activity in noncutaneous afferents

Summary

1) The tactile dermatomes L6—S1 in the cat were mapped by means of mechanical stimulation of the skin and a) single unit recordings in the dorsal root and b) recordings from dorsal rootlets

2) The L6 and L7 dermatomes which are seen as longitudinal stripes on the hindlimb reaching down to the toes are situated on the antero medial and postero-lateral aspects of the limb respectively (Fig 5)

3) Somewhat more medially in relation to the L7 dermatome the S1 dermatome was situated on the posterior aspects of the limb usually reaching to the middle of the foot but sometimes down to the toes (Fig 4)

Results

A ADULT CATS

For comparison the fibre composition of cutaneous and articular nerves in the adult stage is described before the postnatal development of such nerves As can be seen in Fig 6 showing the calibre spectra of cutaneous and articular nerves in an adult cat, the largest fibres of the hindlimb nerves studied were found in nn cut fem lat saphenus and suralis The largest fibres of nn plantaris and per superf were smaller than those of the three nerves mentioned above The size of the largest fibres of n cut fem post was *in between those of the largest fibres in the above two groups* of nerves

The cutaneous branch of n radialis taken in the forefoot contained nearly as large fibres as the nerve containing the largest fibres of the hindlimb

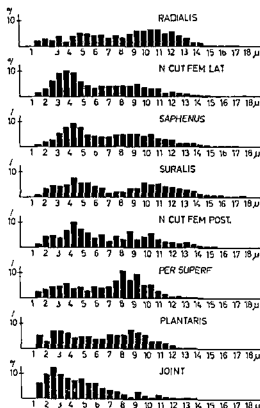


FIG 6 Histograms of calibre spectra of cutaneous nerves and the medial knee joint nerve in an adult cat For further explanation see text

The calibre of the largest fibres of the medial knee joint nerve was within the range of those of the hindlimb cutaneous nerves

The configuration of the histograms of the adult cutaneous nerve fibres can be seen in Fig 6 Cutaneous nerves have two maxima The absolute figures for these maxima and the largest fibres can be seen in Fig 6 and in the Tables An exact description of each spectrum was considered beyond the scope of this study The configuration of the calibre spectra of the cutaneous nerves and that of the articular nerve might however, be compared It can be seen that the medial knee joint nerve had only one maximum (*Skoglund* 1956 *Ekholm* and *Skoglund* 1964a) whereas the cutaneous nerves had two maxima (*Skoglund* and *Romero* 1965)

B KITTENS

The myelinated fibres of the newborn kittens were very small in the cutaneous nerves In fact, it was difficult to stain the thin myelin sheaths (cf *Skoglund* and *Romero* 1965) Perfect sections of all nerves studied in the same kitten were not always obtained in the most immature cases

The following text refers to Figs 7—8 which are histograms of cutaneous and articular nerves of different maturation Four nerves have been chosen for illustration

1) N cut fem lat supplying a skin area on the thigh — a proximal structure (see Chapter II) This is one of the nerves containing the largest fibres in the adult cat

2) N per superf supplying a skin area on the foot — a distal structure (see Chapter II) The largest fibres of this nerve in the adult cat were smaller than those of the other cutaneous nerves except for n plantaris which was similar to n per superf

3) N radialis supplying a skin area on the forefoot (representing a forelimb nerve) and having nearly as large fibres as the largest ones of the adult hindlimb nerves studied

4) The medial knee joint nerve (articular structure)

As can be seen in Fig 7A from a 4 day old kitten the maximum of each calibre spectrum was found in the group of smallest fibres (between 1 21 and 1 76 μ) N cut fem lat had a few (three) fibres that were larger than the largest ones of n per superf and the articular nerve N radialis had a fibre still larger than the largest examples of n cut fem lat

In Fig 7B from a 15 day old kitten the same pattern as in Fig 7A can be seen N radialis had the largest fibres and n cut fem lat had fibres larger than the largest ones of n per superf and the articular nerve

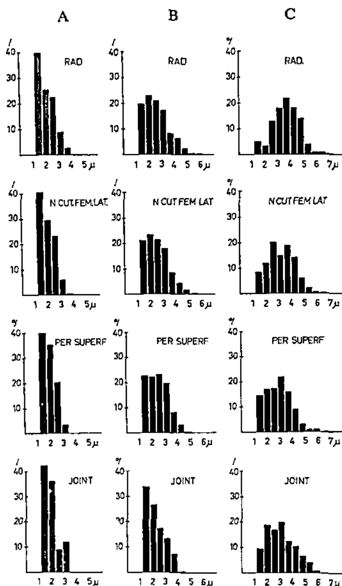


FIG 7 Histograms of calibre spectra of cutaneous nerves and the medial knee joint nerve in 4 (A) 15 (B) and 21 (C) days old kittens

As can be seen in Figs 7C and 8A C from kittens up to 90 days old, the same relations between the largest fibres of the nerves were essentially maintained in kittens up to 65 days old In the 90 day old kitten (and in the adult stage Fig 6) however n cut fem lat had larger fibres than n radialis

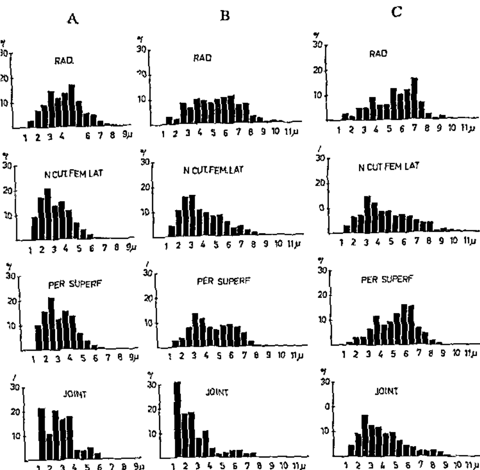


FIG 8 Histograms as in Fig 7 in 45 (A) 65 (B) and 90 (C) days old kittens

In Fig 9 the calibres of the largest fibres of these nerves have been plotted against age It can be seen that in the beginning of the developmental stage up to an age of 65 days n radialis contained the largest fibres After this age n cut fem lat had larger fibres than n radialis It is also obvious that the largest fibres of the medial knee joint nerves never exceeded the diameter of the largest fibres of n cut fem lat The calibres of the largest articular nerve fibres were about the same as n per superf or smaller

In the 21 days old kitten (Fig 7C) and in younger kittens only one maximum appeared in each calibre spectrum In the 45 days old kitten (Fig 8A) however two different maxima were discernible in some cutaneous nerve calibre spectra The two maxima of cutaneous nerve calibre spectra were sometimes seen in the 65 days old and 90 days old kitten as well

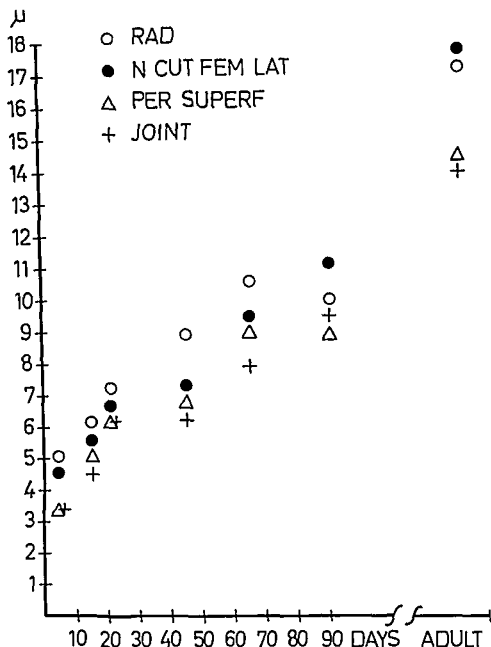


FIG 9 Largest fibre diameter of cutaneous and articular nerves plotted against age *All nerves plotted on a particular age are obtained in one individual animal*

as in the adult cat The articular nerve on the other hand never developed two maxima in its fibre spectrum

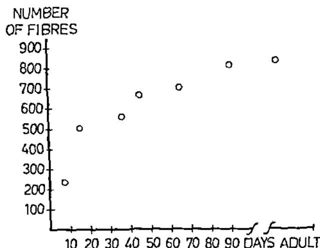


FIG 10 The total number of myelinated fibres in n suralis plotted against age

C TOTAL NUMBER OF MYELINATED FIBRES

It has been pointed out earlier (*Rexed* 1944 *Skoglund* and *Romero* 1965) that the number of myelinated fibres increases during development In the present study n suralis was chosen for such an analysis In Fig 10 the total number of myelinated fibres in the sural nerve are plotted against age The adult number of myelinated fibres was reached at an age of about 2—3 months

Discussion

Before discussing the comparisons between the calibre spectra some comments on the method should be made As has been pointed out earlier (*Skoglund* and *Romero* 1965) the correlation between chronological age and the actual maturity of the nervous system varies considerably between different kittens Thus it has to be taken into consideration that the fibre composition of comparable nerves and the size of the largest nerve fibres of such nerves in different animals of the same age varies over a rather wide range It is evident that comparisons between calibre spectra within a series of animals must be made with certain precautions when each age is represented by only one animal However comparisons between different nerves within the same animal can be made with more certainty The inter individual differences are then no problem but the variations in the methodological errors have to be considered When comparisons with other investigations are made methodological differences must be considered

The technical quality of the material is of great importance, if the sections are bad satisfactory measurements cannot be made This is a problem especially when using material from young animals One of the efforts made here to obtain good technical quality, was fixation of the material in an isotonic buffer considered to give minimum deterioration of the structure (*Sjostrand* 1953) Furthermore all material not successfully treated was discarded All measurements were made by one person (the author) and in accordance to certain rules (see Methods) When using this fixation a somewhat irregular form of cross sectioned nerve fibres is *obtained A relatively high primary magnification in the photomicroscope* was used in order to obtain high quality photocopies of low secondary magnification

Skoglund and *Romero* (1965) showed a proximo distal development of muscle nerves For direct comparisons to be made, with any degree of accuracy, between different nerves with regard to their maturation at a certain developmental stage it seems to be a prerequisite that their largest fibre diameters either become eventually of the same order of size or *that if they show an ultimate difference they will also show a difference* in the opposite direction during some stage of the development However comparisons can also be made between the relative stages of maturation of different nerves that is to say the number of times the different nerves have to increase their largest fibre diameter to reach their adult stage, regardless of whether they eventually become similar in size or different one way or the other

One aim was to compare cutaneous nerves innervating proximal and *distal structures directly e g nn cut fem lat and per superf The largest* fibre diameters of these nerves differ during the postnatal development but they also differ in the same direction in the adult stage Thus direct comparisons at different stages of development could not be made A relative value of maturation was obtained however and it was found that in the 4 days old kitten the distal nerve (n per superf) had to increase its largest fibre diameter 4 23 times to reach the adult size whereas the proximal nerve (n cut fem lat) in spite of the fact that it eventually reached larger diameters *in the adult stage than the distal nerve only had to increase* its largest diameter 3 92 times In this sense the proximal nerve appears to be relatively more mature than the distal nerve just after birth A similar calculation for the articular nerve gave an increase of 4 06 times This nerve which supplies a different structure lying between that of the proximal and the distal cutaneous nerves thus shows a developmental value lying in between those for the cutaneous nerves Using the material obtained by *Skoglund* and *Romero* (1965) the same calculation can be made for

a proximal (adductor) and a distal muscle nerve (gastrocnemius med) The largest diameter of the distal nerve increases 4 35 times from the newborn to the adult stage whereas that of the proximal nerve increases only 4 06 times

Thus the results obtained by comparisons of the calculated relative stages of maturation between proximal and distal nerves supplying different types of structures are consistent with the finding of a proximo-distal development in the muscle nerves (*Skoglund* and *Romero* 1965) obtained by means of direct comparisons

The finding that n radialis of the forelimb had larger fibres than any of the hindlimb nerves up to 65 days and that after this age the largest fibres were smaller than the largest fibres of the hindlimb nerves might indicate that the forelimb nerves are more highly developed at birth and for some time afterwards (cf *Skoglund* and *Romero* 1965) This is in accordance with a cephalo-caudal development (*Kingsbury* 1932) N radialis increases its largest diameter 3 39 times from the age of 4 days to the adult stage This value is lower than those of the hindlimbs nerves investigated here and indicates in accordance with the direct comparison that n radialis is more mature than the hindlimb nerves just after birth

The fibre analysis of the medial knee joint nerve during the postnatal development which has not been made previously, shows that the calibres of the largest fibres of most knee joint nerves were about the same as n per superf The largest fibres of the joint nerves were never larger than those of the cutaneous nerves containing the largest fibres of the hindlimb *Skoglund* (1956) showed that in adult cats there is normally a considerable variation in the sizes of the largest fibres of articular nerves This is probably due to the different pathways available for the knee joint nerve fibres to reach the spinal cord the medial nerve comprises only a part of the fibres

In connection with physiological experiments on the knee joint in the cat (*Ekholm* and *Skoglund* 1960 *Ekholm* *Eklund* and *Skoglund* 1960) the author has earlier studied the calibre spectra of adult medial knee joint nerves (*Ekholm* and *Skoglund* 1964a) The wide variation found by *Skoglund* (1956) was confirmed and it was shown that sympathetic nerve fibres probably do contribute but fairly little to the groups of small myelinated fibres of the knee joint nerve The fibre spectrum of the adult medial knee joint nerve of the adult cat shown in the present investigation is in line with those of the nerves containing the relatively large fibres in the earlier investigation mentioned above This fibre spectrum is thus suitable for comparison with the material from young kittens The calibre spectra of n saphenus and n suralis were essentially in accordance with those obtained by *Skoglund* and *Romero* (1965)

Table I The calibre spectrum of n radialis

Animal No	1.21–1.76	1.76–2.32	2.32–2.87	2.87–3.42	3.42–3.97	3.97–4.52	4.52–5.08	5.08–5.63	5.63–6.18	6.18–6.73	6.73–7.28	7.28–7.84	7.84–8.39	8.39–8.94	8.94–9.49	9.49–1[illegible]
156	231	148	130	52	16	2	1									
130	175	152	159	66	14	5	1	1								
154	172	201	191	154	80	53	22	4	1							
196	26	17	65	91	111	93	71	20	6	6	1					
131	43	111	149	237	189	217	271	170	134	77	31	12	4	1		
201	25	13	52	41	63	58	53	61	66	69	46	48	18	12	3	[illegible]
133	13	8	29	27	46	30	30	63	52	60	81	34	12	4	7	[illegible]
149	14	19	18	30	14	31	39	36	31	32	25	30	25	36	46	[illegible]

Table II The calibre spectrum of n cut fem lat

Animal No	1.21–1.76	1.76–2.32	2.32–2.87	2.87–3.42	3.42–3.97	3.97–4.52	4.52–5.08	5.08–5.63	5.63–6.18	6.18–6.73	6.73–7.28	7.28–7.84	7.84–8.39	8.39–8.94	8.94–9.49	9.49–1[illegible]
156	220	159	127	33	2	1										
130	182	156	126	102	58	4	4	6								
154	181	199	183	155	75	42	14	2								
196	62	88	148	114	138	106	46	18	8	2						
131	127	231	285	188	204	155	93	55	29	6	1					
201	28	68	102	106	67	57	53	55	37	22	24	15	8	3	1	
133	18	38	44	89	73	54	54	40	44	39	30	24	24	6	11	[illegible]
149	29	56	120	175	191	172	112	84	61	78	77	77	78	82	90	[illegible]

Table III The calibre spectrum of n saphenus

Animal No	1.21–1.76	1.76–2.32	2.32–2.87	2.87–3.42	3.42–3.97	3.97–4.52	4.52–5.08	5.08–5.63	5.63–6.18	6.18–6.73	6.73–7.28	7.28–7.84	7.84–8.39	8.39–8.94	8.94–9.49	9.49–1[illegible]
156	307	273	181	46	1											
130	44	115	151	100	40	5	1									
196	47	56	76	87	73	60	32	15	3							
131	251	313	373	261	254	276	263	161	107	41	14	4	1			
201	41	53	76	61	48	48	52	63	63	52	31	14	9	2	1	
133	20	27	45	81	57	58	53	75	62	46	26	28	9	13	4	[illegible]
149	29	63	94	175	241	286	245	155	138	127	132	152	155	162	164	[illegible]

Table IV The calibre spectrum of n suralis

Animal No	1.21–1.76	1.76–2.32	2.32–2.87	2.87–3.42	3.42–3.97	3.97–4.52	4.52–5.08	5.08–5.63	5.63–6.18	6.18–6.73	6.73–7.28	7.28–7.84	7.84–8.39	8.39–8.94	8.94–9.49	9.49–1[illegible]
130	36	85	80	23	6	2	1									
154	147	105	107	74	47	21	6	1								
172	26	37	64	137	122	106	57	6	3	2						
131	54	80	104	88	82	108	75	41	23	7	4	1				
201	44	49	79	68	53	42	45	88	85	77	43	16	10	5		
133	18	30	67	77	99	63	69	87	88	69	71	37	14	14	6	[illegible]
149	9	28	39	36	43	65	50	50	37	32	15	19	23	30	27	[illegible]

04- 60	10 60- 11 15	11 15 11 70	11 70 1 .25	12..5 1..80	12 80- 13 36	13 36- 13 91	13 91 14 46	14 46- 15 01	15 01 15 56	15 56- 16 12	16 1? 16 67	16 67 17 2..	17 .. 17 77	Number of fibres measured
														580
														573
														878
														507
														1646
1														634
														498
50	49	37	43	31	24	11	11	3	4	4	1	1		742

04- 60	10 60- 11 15	11 15- 11 70	11 70- 12.25	12 25 12 80	12 80- 13 36	13 36- 13 91	13 91 14 46	14 46- 15 01	15 01 15 56	15 56- 16 12	16 12 16 67	16 67 17 ..	17 22 17 77	Number of f bres measured
														542
														638
														851
														730
														1374
														646
3	1													598
61	65	32	36	25	23	11	12	5	6	1	0	1	2	1830

0 04- 0 60	10 60- 11 15	11 15 11 70	11 70- 12.25	12 25 12.80	1 80- 13 36	13 36 13 91	13 91 14 46	14 46 15 01	15 01 15 56	15 56 16 12	16 1 16 67	16 67 17 2	17 2.. 17 77	Number of fibres m a urec
														808
														456
														449
														2319
														614
														607
.60	111	90	83	44	46	43	36	20	10	7	3	1		3110

0 04- 10 60	10 60 11 15	11 15 11 70	11 70 12 25	12 ..5 1 80	1..80- 13 36	13 36- 13 91	13 91 14 46	14 46 15 01	15 01 15 56	15 56- 16 1	16 1 16 67	16 67 17 2..	17 2.. 17 77	Number of f bres me ured
														233
														233
														560
														667
														704
I														813
44	45	38	34	28	26	21	11	8	6	6	1	1	7	829

Table V The calibre spectrum of n per superf

Animal No	1.1–1.76	1.76–2.32	2.32–2.87	2.87–3.4	3.4–3.97	3.97–4.52	4.52–5.08	5.08–5.63	5.63–6.18	6.18–6.73	6.73–7.28	7.28–7.84	7.84–8.39	8.39–8.94	8.94–9.49	9–10
156	256	226	133	22												
130	86	169	142	44	10											
154	215	218	220	187	78	29	2									
196	110	88	132	163	120	68	24	2	2							
131	130	204	278	163	198	176	85	44	23	4						
201	18	26	57	97	83	53	81	100	101	58	38	14	4	2		
133	7	19	20	39	70	49	56	75	98	93	39	27	9	3		
149	14	22	29	30	36	24	27	19	26	25	27	42	66	44	59	3[illegible]

Table VI The calibre spectrum of n plantaris

Animal No	1.1–1.76	1.76–2.3	2.3–2.87	2.87–3.42	3.42–3.97	3.97–4.52	4.5–5.08	5.08–5.63	5.63–6.18	6.18–6.73	6.73–7.28	7.28–7.84	7.84–8.39	8.39–8.94	8.94–9.49	9–10
156	135	127	106	22												
154	290	222	233	213	133	63	9									
196	101	75	123	152	109	56	22	2	1							
201	51	49	43	57	50	57	91	99	71	36	8	3	2			
149	47	30	60	59	52	36	42	41	33	34	47	44	45	64	52	48

Table VII The calibre spectrum of n cut fem post

Animal No	1.1–1.76	1.76–2.3	2.3–2.87	2.87–3.4	3.4–3.97	3.97–4.52	4.5–5.08	5.08–5.63	5.63–6.18	6.18–6.73	6.73–7.28	7.28–7.84	7.84–8.39	8.39–8.94	8.94–9.49	9–10
156	49	41	32	4												
201	26	27	70	60	40	27	39	59	44	34	16	4	2	1		
149	8	26	29	22	41	61	44	35	23	29	14	25	22	40	19	25

Table VIII The calibre spectrum of the medial knee joint nerve

Animal No	1.21–1.76	1.76–2.3	2.3–2.87	2.87–3.42	3.4–3.97	3.97–4.52	4.52–5.08	5.08–5.63	5.63–6.18	6.18–6.73	6.73–7.28	7.28–7.84	7.84–8.39	8.39–8.94	8.94–9.49	9–10
185	12	11	10	10												
156	14	12	3	4												
199	13	6	4	0	2	1										
182	14	12	24	12	2	6										
154	33	76	17	13	7	1										
196	9	18	16	19	12	10	6	4	1							
179	10	27	17	26	18	12	3	8	3							
172	7	9	10	7	5	6	4	8	1							
131	36	18	34	28	29	6	6	8	4							
201	45	26	27	11	16	5	2	3	4	4	2	2				
200	5	5	9	14	17	21	17	10	9	8	4	3	6	0	2	
133	8	17	31	27	22	17	18	12	9	7	4	4	5	2	1	
149	18	28	37	29	24	22	23	19	19	13	10	9	6	2	9	4

10 60- 11 15	11 15 11 70	11 70- 12.25	12.25 12 80	12 80 13 36	13 36- 13 91	13 91 14 46	14 46- 15 01	15 01 15 56	15 56- 16 12	16 12 16 67	16 67 17 ..	17 22 17 77	Number of fibres measured
													637
													451
													949
													752
													1305
													732
													604
20	11	4	5	2	1	1							600

10 60 11 15	11 15- 11 70	11 70- 12. 5	12 25 12.80	12 80- 13 36	13 36- 13 91	13 91 14 46	14 46- 15 01	15 01 15 56	15 56 16 12	16 12 16 67	16 67 17 .2	17 2. 17 77	N mber of fib es measured
													390
													1163
													641
													617
26	23	11	3	1	1								825

10 60- 11 15	11 15 11 70	11 70 12 25	12..5 12.80	12 80 13 36	13 36 13 91	13 91 14 46	14 46- 15 01	15 01 15 56	15 56- 16 1	16 12 16 67	16 67 17	17.. 17 77	Number of fib es m asured
													126
													449
19	16	13	12	14	9	5	2	1					590

10 60 11 15	11 15 11 70	11 70- 12 25	1.. 5 12 80	1..80 13 36	13 36- 13 91	13 91 14 46	14 46 15 01	15 01 15 56	15 56- 16 1.	16 12 16 67	16 67 17 ..	17.22 17 77	Number of f bres measured
													43
													33
													26
													70
													97
													95
													124
													57
													169
													147
													130
5	3	2	1	2	1								184
													288

Summary

1) The postnatal development of the fibre composition (calibre spectra) of cutaneous and articular nerves was studied in osmium stained sectioned material For comparison data from adult material was also presented

2) In newly born kittens the myelinated fibres of cutaneous nerves were very small The diameter range of the nerve fibres increases during the postnatal development and the increase in largest fibres at an age of 90 days was about 40—50 % of the total increase between birth and the adult stage

3) The following relationships between the largest fibres in some of the cutaneous nerves studied were maintained up to an age of 65 days N radialis (forefoot) contained the largest fibres n cut fem lat (proximal structure of hindlimb) had larger fibres than n per superf (distal structure of hind limb) At a later age and in the adult stage n cut fem lat had larger fibres than n radialis The change of the relationship between the forelimb and hindlimb nerves is consistent with a cephalo-caudal development and a calculation of the relative maturation of proximal and distal cutaneous hindlimb nerves gave values consistent with a proximo-distal development

4) The size of the largest fibres of the medial knee joint nerve was about the same as that of fibres in n per superf and never exceeded the largest fibres of n cut fem lat

CONDUCTION VELOCITY OF CUTANEOUS NERVES DURING POSTNATAL DEVELOPMENT

Introduction and literature

A relationship between conduction velocity and fibre diameter was reported by *Hursh* (1939 a) — the well known factor of six *Hursh* (1939 b) also studied the properties of growing nerve fibres in cats and kittens The conduction velocity of cutaneous nerves (n saphenus) increased from 11 m/sec at an age of 4 days to 60 m/sec at 87 days The increasing conduction velocities were plotted against the increasing length of the leg during the development The relationship was clearly a linear one *Hursh* concluded that the movements of the kittens are not slowed because of the lower conduction rate since the latter is nicely compensated for by the decreased length of the nerve path

As pointed out by *Skoglund* (1960 b) this conclusion cannot be drawn from *Hursh s* material The conduction time for a stretch of nerve of the same length as the limb can actually be calculated from *Hursh s* material to decrease with increasing length of the limb As for muscle afferents *Skoglund* (1960 b) calculated that 80 % of the decrease in conduction time has already occurred when the conduction velocity has reached 30 m/sec (at an age of about 20 days) For comparison with the muscle afferents he also presented some conduction velocity values from the sural nerve and showed that here the conduction velocity increased more slowly than that of the muscle nerves (0 65 m/sec per day compared with 0 99 m/sec per day)

In the present work the postnatal changes in the conduction velocity of cutaneous nerves have been studied with special regard to the following relationships

1) Conduction velocity — chronological age
2a) Conduction velocity — increase in length of limb
2b) Conduction time — increase in length of limb
3) Conduction velocity — fibre spectra (Chapter V)

Material and Methods

The conduction velocity of the sural nerve was usually determined in connection with other physiological experiments A large number of values were obtained from kittens and cats ranging in age from newborn to adult Pentobarbital was used for anaesthesia A lumbo sacral laminectomy was performed and the dorsal roots L6 L7 and S1 were freed and sectioned near the spinal cord (for details of the method see Chapter III) The sural nerve was stimulated with squarewave shocks having a duration of 0 3 msec and a strength supramaximal to the first potential wave recorded The potentials were recorded from one of the appropriate dorsal roots (see Chapter III) and photographed on bromide paper

The conduction velocity of the sural nerve was calculated in the following way The conduction time was measured on the bromide paper from the beginning of the shock artifact to the rise of the first potential wave The conduction distance was measured from the cathode of the stimulating electrode to the recording electrode on the dissected nerve In some cases the same nerve in which the conduction velocity had been determined was taken out for staining and fibre analysis (Chapter V) Postmortally the maximal length of the femur was measured from the top of the femoral head to the distal end of the condyles

Results

In Fig 11 the conduction velocity of the sural nerve is plotted against age It can be seen that in newborn kittens the conduction velocity varied between 8—12 m/sec and increased with increasing age by approximately 0 6 m/sec per day The equation of the linear regression line calculated by the method of least squares is $y = 0\,58x + 10\,9$ This value is similar to though slightly smaller than that (0 65 m/sec per day) given by *Skoglund* (1960 b)

The conduction velocity was then plotted against the length of the femur (Fig 12) The linear regression equation (method of least squares) is $y = 1\,03x - 14\,2$ It shows the trend of the development which may be expressed as follows The conduction velocity approximately increases by 1 m/sec per 1 mm increase in the length of the femur from 10 m/sec and a 25 mm femur in the newborn stage (up to a stage when the femur is 60 mm long)

The conduction time for a stretch of nerve of the same length as the femur was calculated from the regression line in Fig 12 and plotted in Fig 13 When the femur was 25 mm the conduction velocity was 10 8

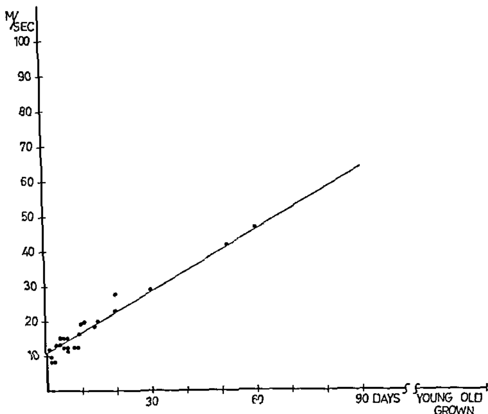


FIG. 11 Conduction velocity of n suralis plotted against age See text

m/sec and the conduction time 2 31 msec whereas the conduction time was 1 37 msec at a stage when the femur was 60 mm and the conduction velocity 45 8 m/sec It can be seen that the conduction time decreases with increasing femur length

The graph shown in Fig 12 and the linear regression line can be used when a rough estimate of the conduction velocity is desired without the use of electrophysiological measurements For practical purposes a simple formula was calculated Since the slope is very near unity only one constant has to be used (y=x—14) It follows that the length of the femur expressed in mm minus 14 gives the numerical value of the conduction velocity of the sural nerve (m/sec) with an accuracy of about ± 5 m/sec (this error was not calculated statistically but estimated by eye)

For purely practical purposes a graph (Fig 14) is shown in which the length of the femur is plotted against age This graph can be used when it is desired to estimate the age of the animal

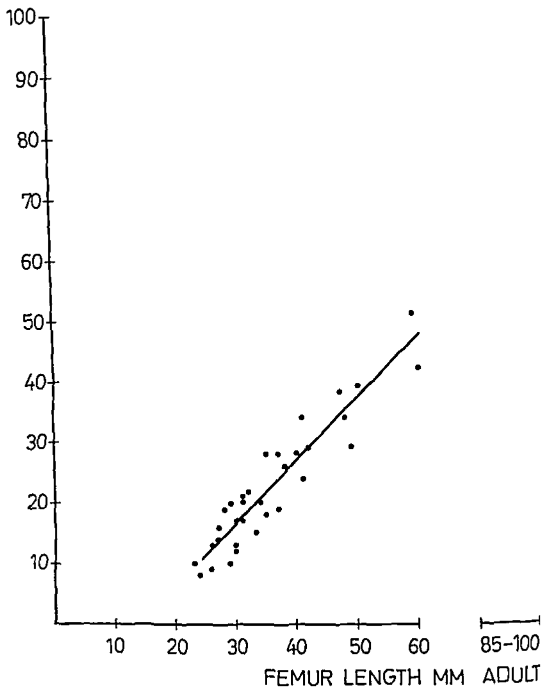


FIG 12 Conduction velocity of n suralis plotted against femur length See text

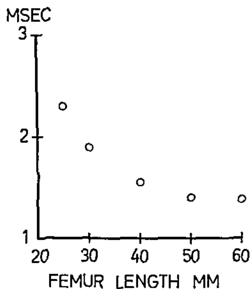


FIG 13 Conduction time for a stretch of nerve (suralis) of the same length as the femur plotted against femur length

EMUR LENGTH
MM
00
90
80
70
60
50
40
30
20
10
10 20 30 40 50 60 DAYS ADUL

FIG 14 Length of femur plotted against age

Table IX Quotient between conduction velocity in the largest fibres and fibre diameter in n suralis

Animal No	Conduction velocity m/sec	Largest fibre diameter ( )	Quotient cond vel /diam
1	22 0	4 52	4 9
2	22 8	5 08	4 5
3	29 3	6 18	4 7
4	29 5	6 73	4 4
5	39 5	7 84	5 0
		average	4 7

The relationship between conduction velocity and fibre size has been investigated earlier experimentally Since however, the experimental conditions may vary from one investigation to another, it cannot be expected that the relationship found here is strictly the same as those obtained in other experiments In order to provide a rough check of the method used in the present investigation a fibre analysis was made on the same sural nerve in which the conduction velocity was determined In Table IX it can be seen that when the conduction velocities are divided by the maximum fibre diameter of the calibre spectrum (largest diameter of the group of largest fibres) factors are obtained varying from 4 4—5 0, with an average of 4 7 This value can be used for a rough calculation of the fibre spectrum corresponding to a certain conduction velocity (see Discussion)

Discussion

The postnatal increase of 0 6 m/sec per day in the conduction velocity of the sural nerve found in this study is consistent with but somewhat smaller than that observed by *Skoglund* (1960 b)

The conduction velocity has been correlated with the femoral increase and it was found that the velocity increased by approximately 1 m/sec per 1 mm increase of the femur The calculated conduction time for the stretch of nerve of equal length to the femur decreases with increasing conduction velocity and increasing femoral length It follows that in young animals the slow conduction is not completely compensated by the shorter impulse pathway This confirms the calculations made by *Skoglund* (1960 b) but disagrees with the conclusion drawn by *Hursh* (1939 b)

In laboratory work with kittens it is often a problem to determine the

age of the animal and its degree of nervous maturation *Skoglund* (1960 c e) used conduction velocity as a measure of nervous maturity The gestation period is reported by *Scott* et al (1957) to be 65±4 days In the present investigation a graph and a simple formula (y=x—14) are given by which a rough calculation of the conduction velocity in the sural nerve can be made from a knowledge of the length of the femur Another graph is given from which the age of the animal (if unknown) can be calculated from the length of the femur

Two regression lines have been calculated It may be argued that the best fit of the values is not given by a straight line In fact the values presented in Fig 12 give the impression of a somewhat curvilinear relationship The straight regression lines however, have been given to illustrate the general trend of the development Although they may not be those best fitted to the experimental values the conclusions drawn from them seem valid

The ratio between conduction velocity and fibre diameter was 4 7 This figure is obviously restricted to the experimental conditions of the present investigation Several possible errors are involved The conduction time is measured to the rise of the first potential wave This point on the neurogram is easily distinguishable and offers a stable and clear point for measurements A few very large fibres (see Chapter V) may cause an early deflection of the neurogram, but this deflection is ill defined and thus not suitable for measurements The method for measuring time used here thus gives lower values for conduction velocity than the true values When evaluating the ratio between conduction velocity and fibre size several other factors must also be taken into consideration e g the actual point of stimulation the measurement of the length of the dissected nerve the temperature and the morphological technique

The correlation factor obtained here (4 7) is lower than the value of six given by *Hursh* (1939 a) The regression line calculated from *Hursh s* (1939 a) material has a slope of 6 0 which means that the velocity in meters per second can be obtained by multiplying the diameter in micra by this factor However *Hursh s* relationship appears to be expressed by the following equation

$$\text{conduction velocity} = 6 \times \text{fibre diameter} - 4 \qquad (1)$$

Thus simply multiplying fibre diameter by the factor of six should give an estimate of the conduction velocity which is 4 m/sec too high The fibre diameter can be calculated from the conduction velocity since from (1)

$$\text{fibre diameter} = \frac{\text{conduction velocity} + 4}{6} \qquad (2)$$

Again if the constant (4) is omitted an error is introduced which is proportionally greatest in the small diameters

In *Hursh s* material the conduction velocity for fibres 4 μ and 8 μ in diameter were 20 and 42 m/sec respectively i e the velocity diameter ratio was about 5 This is in fairly good agreement with the mean value of 4 7 obtained in the present investigation and seems to be a more correct factor than that suggested by *Hursh* (1939 a) especially for low conduction velocities

As will be further pointed out in chapter VII the stage at which the conduction velocity is 20 m/sec is of particular interest With the aid of the factor 4 7 the diameters of the largest fibres of such nerves can be calculated to be about 4 3 μ If this diameter is compared with those of the calibre spectra of the sural nerves in the 14 and 21 day old kittens (the age at which the conduction velocity of 20 m/sec is attained) given in chapter V, it can be seen that these nerves have a few somewhat larger fibres but that there are a considerable number of fibres of about 4 3 microns The 45 day stage is also of interest with regard to the postnatal changes in the physiological properties of the cutaneous nerve fibres (Chapter VII) The conduction velocity is then about 37 m/sec The calculated diameter of the largest fibres is about 8 μ, which fits well with the findings in Chapter V

Summary

1) The conduction velocity of the sural nerve was determined in kittens of different ages

2) The conduction velocity increased by approximately 0 6 m/sec per day from approximately 10 m/sec at birth

3) The conduction velocity increased by approximately 1 m/sec per one mm increase of the femur postnatally

4) The conduction time for a stretch of nerve equal to the length of femur decreased postnatally

5) For practical purposes a formula was calculated which can be used for a rough estimation of the conduction velocity (in n suralis) from the femur length conduction velocity (m/sec) = length of femur (mm) minus 14

6) When the conduction velocity (m/sec) of some sural nerves was divided by the diameter (μ) of the largest fibres of the calibre spectra obtained from the same nerves an average quotient of 4 7 was obtained

7) Using *Hursh s* (1939 a) own graph it was found that the conduction velocity cannot be obtained correctly by only multiplying the fibre diameter

with the factor of 6 The equation should be conduction velocity (m/sec) $= 6 \times$ fibre diameter (μ) — 4 When the factor of 6 alone is used the error introduced is greatest for small fibres When calculating from *Hursh s* data the factor for small fibres should be about 5

8) The largest fibre of a nerve giving a conduction velocity of 20 m/sec was calculated to be 4 3 μ by means of the factor 4 7 (the corrected *Hursh* equation gives 4 μ)

CHAPTER VII

DISCHARGE PATTERNS OF CUTANEOUS AND ARTICULAR SENSE ORGANS DURING POSTNATAL DEVELOPMENT

Introduction and literature

Nerves course beneath the (superficial) epithelium and end under it in primitive free terminations before any reflexes can be elicited in mammalian embryos (*Windle* 1940) A morphological basis for cutaneous nerve activity is evidently established early in development The function of skin afferents during ontogenesis has been studied in embryos and fetuses (for ref see *Windle* 1940 *Carmichael* 1951 1954) In all these studies the following procedure was used the skin was stimulated and it was observed at what age a reflex movement appeared The test situation used thus requires function in all parts of the reflex arc and does not give information about the sequence in which different parts of the reflex arc develop However, since reflex movements are elicitable by mechanical (and thermal) stimulation of the skin in cat embryos (*Windle* and *Griffin* 1931) the sensory part of the reflex arc must function early in development

The sensory areas or reflexogenous zones in the skin appear first in the head neck and forelimbs and extend caudally with increasing age The last such zones to be established are those of the hindlimb and tail which however seem to exist before full term in kittens (*Windle* 1940) It should thus be possible to evoke activity from the cutaneous sense organs in these regions already at birth

In this investigation the activity arising from the sense organs in the skin has been recorded from both peripheral nerves and single functioning dorsal root fibres in an attempt to analyze the discharge pattern during postnatal development For comparisons with the exteroceptors of the skin the deve lopment of the discharge pattern in joint proprioceptors was also studied

The activity of proprioceptors in kittens was studied by *Skoglund* (1960 1963) who found that the discharge of receptors in the gastrocnemius muscle in response to stretch was phasic in newborn and young kittens Tonic responses were obtained from proximal muscles at the same age With increasing age more sustained discharges were obtained from the gastrocne

mius muscle but the frequency of the discharge was low The responses of Golgi tendon organs were also phasic in newborn kittens At the stage when the muscle nerves have reached a conduction velocity of 20 m/sec and the absolute refractory period has attained adult values tonic discharges from the muscle receptors were found It was suggested that the appearance of tonic discharges might be related to parallel changes in the electrical properties of the nerve fibres and the mechanical properties of the muscle

There is a complete lack of knowledge as regards the development of the discharge patterns from cutaneous afferents The aim of the present investigation was twofold 1) to ascertain if there is the same relation between discharge pattern and conduction velocity in the exteroceptors in the skin as in the proprioceptors 2) to study the development of the discharge patterns of different types of cutaneous receptors to obtain the information necessary for an evaluation of the afferent link of the reflexes that can be elicited from the skin Some of the findings reported here have been published in a preliminary note (*Ekholm* 1965)

To compare the properties of immature and mature sense organs, earlier experiments on sense organs in the adult cat have been repeated and in some respects extended There have been numerous investigations since *Adrian* and *Zotterman* (1926a b) succeeded in obtaining recordings from afferent nerve fibres and describing the discharge characteristics of individual sense organs Much information is now available concerning different types of cutaneous afferents (for ref see *Granit* 1955 *Hensel* 1966 a b *Iggo* 1966) Earlier observations on cutaneous sensory units in adult cats have been summarized in connection with the description of the present results in the immature animals Joint proprioceptors of adult cats have been studied by *Andrew* and *Dodt* (1953) *Boyd* and *Roberts* (1953) *Boyd* (1953 1954) *Skoglund* (1956) and *Eklund* and *Skoglund* (1960)

In the present investigation of the postnatal development of cutaneous sense organs two types of cutaneous myelinated afferent units of hairy skin have been studied 1) hair follicle afferent units (hair units) 2) slowly adapting mechano sensitive afferent units (touch units) (*Iggo* 1966)

The following specific questions have been treated 1) Is the activity of the sense organs in young animals similar to that in adults? 2) If not in what way does it differ? 3) What is the frequency adaptation number of impulses etc obtained in response to adequate stimulation of the immature sense organs? 4) Can such parameters be correlated to the maturity of the nerve fibres in terms of conduction velocities? 5) Are there differences in the development of the function of exteroceptors in the skin and the proprioceptors in muscles and joints?

Material and Methods

The results from 95 cats and kittens anaesthetized with pentobarbital (40 mg/b w i p) are presented here Usually the hairs of the hindlimbs were clipped down to a height of about 1 mm After a lumbo sacral laminectomy the dorsal roots L4 L5 L6, L7 and S1 were dissected free and cut near the spinal cord To avoid interference with activity from muscle and tendon sense organs the following muscle nerves were cut in the hindlimb the hamstring tibial peroneal and femoral (except n saphenus) An afferent unit consists of a nerve fibre its terminals and associated non nervous elements (*Iggo* 1966) The fibres of the units were isolated in the dorsal root by dissecting free thin filaments under the dissecting microscope Because of the size of the fibres this was a difficult and time consuming work Usually recordings were made from filaments containing only a single active cutaneous afferent but sometimes filaments containing 2—3 afferents were used Sometimes only very few single units could be analyzed in the young animal

In another type of experiment activity in a peripheral nerve was recorded either from the whole nerve or from a dissected thin strand of it N suralis was often used this was dissected free for as long a stretch as possible in the popliteal fossa The medial knee joint nerve (*Gardner* 1944 *Skoglund* 1956) was used for recording from joint sense organs

Mechanical stimulation of the skin was performed with the fingers a pair of forceps a sable brush or a probe In many experiments on the slowly adapting units constant stimulation was performed by applying a spring clip over the receptive skin area In order to produce constant stimuli of different intensity strong and weak clips were used the same strong clip and the same weak clip being used in all the animals which were stimulated in this way

Electrical stimulation was applied by a pair of needles inserted in the skin over the receptive field Although not proved it is highly probable that in this case the nerve fibres or their terminations are stimulated directly The duration of the impulses was 0 1 msec delivered at different frequencies in short trains (up to 10 sec) or for a longer period of time This method is of course liable to error especially if threshold stimuli are used since the stimulation may cause large alterations in the skin resistance

Temperature changes were induced by cooling with ice ether or ethyl chloride and by warming with a lamp The knee joint receptors were stimulated by passive extension and flexion or by a probe pressed against the joint capsule

For temperature control and protection of exposed tissue references should be made to Chapter III The fixation of the animals was generally achieved by threads attached to the skin and a clamp around the base of the tail leaving one hindlimb completely free for localization identification and stimulation of the receptive fields in the skin

The position and shape of the receptive fields of the units were mapped directly on the skin The area of the receptive field of a hair unit was calculated from two diameters measured at right angles ($r_1 \times r \times \pi$)

Recording A nerve filament or a whole nerve was put on a silver hook a reference electrode was inserted into inactive tissue Different amplification systems (see Chapter III) with monitoring by both loudspeakers and oscillo scopes were used The activity was photographed on moving bromide paper either with the oscilloscope beam sweep disconnected and with the camera at maximal speed or with sweep displays To be able to resolve nerve spikes of high frequency it was sometimes necessary to feed the record into a high speed tape (Revox tape recorder) The tape was afterwards played back at low speed and the record displayed on an oscilloscope and photo graphed A sine wave generator or a piezo-crystal indicated time on a second oscilloscope beam

When measuring the maximal frequencies obtained in response to mechanical stimulation of touch units the number of spikes per period of time (50 msec) was counted, during this period more spikes were produced in the adult cats and older kittens (because of their high frequencies) than in young kittens Since this might introduce an error when estimating the frequency the minimum interval between two spikes in the spike train was also measured This gives a more exact value of the frequency over a short interval The adapted frequency was measured as number of spikes per 1 sec

In a series of experiments the conduction velocity of each isolated single unit was determined Only units with their receptive fields in the sural area were used Stimulation electrodes were applied to the undivided sural nerve in the popliteal fossa This procedure however was found to damage the nerve fibres in the young kittens and many units were lost Therefore in most cases the conduction velocity of the whole sural nerve was determined at the end of the experiment (see Chapter VI) and/or a specimen of the sural nerve was removed for fibre analysis (see Chapter V)

Results

A RECORDING FROM THE SURAL NERVE

Recordings were made from the sural nerve in the popliteal fossa the

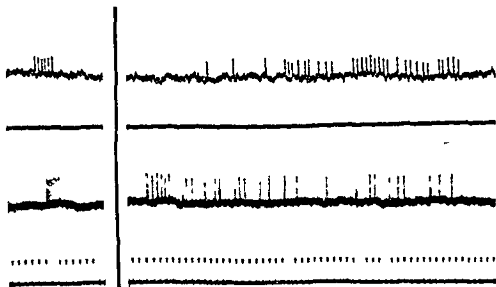


FIG 16 Recordings from single hair units in response to rapid and slow strokes of a brush over the receptive fields in (A) 5 and (B) 45 days old kittens (Spikes retouched) Time 10 msec

kittens No attempt was made to study further the adaptation of the hair units in the kittens When the hairs within the receptive fields were moved the unit responded by a train of impulses The spike frequency in the train on the other hand was studied

Mean frequency of impulse train The hairs over a receptive field were bent by the stroke of a brush and the unit responded with a train of impulses As is illustrated in Fig 16 the mean frequency of the train was lower and the duration of the discharge longer (the number of impulses greater) when the stroke was slow, than if the stroke was rapid By increasing the rapidity of the stimulus the mean frequency could be increased but only up to a certain limit which depended on the age of the animal as is also illustrated in Fig 17 The highest mean frequencies obtained in a series of stimulations with rapid strokes over the receptive fields were plotted as a function of age The unit that was found to give the highest mean frequency in each animal was selected In Fig 17 it can be seen that the maximal mean frequency of the hair unit response is low in young kittens and increases with increasing age to reach values in the adult range some 6 weeks after birth In order to include also

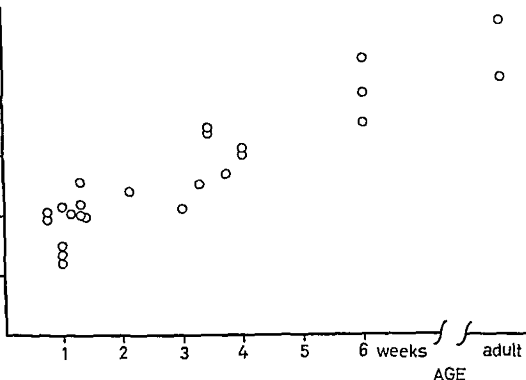


FIG 17 The highest mean frequency recorded from single hair units in response to rapid strokes of a brush over the receptive field plotted against age The unit that was found to give the highest mean frequency (in spike trains of > 3 spikes) in each animal was selected

responses of only 2 spikes in the material the minimum interval found between 2 spikes regardless of the number of spikes in the response was plotted against age (Fig 18) The same trend as in Fig 17 was obtained The interval was of relatively long duration in the early ages and decreased with increasing age to attain adult values at about 6 weeks

The results show that in immature hair follicle units the capacity to produce high frequency responses is limited and that this capacity increases with increasing age

Electrical stimulation When electrically stimulating the receptive fields it was found that the maximal frequency at which a one to one stimulus response relation was maintained was low in young kittens and increased with increasing age

Repeated stimulation Attempts were made to ascertain whether the response of hair units changed when they were stimulated repeatedly by

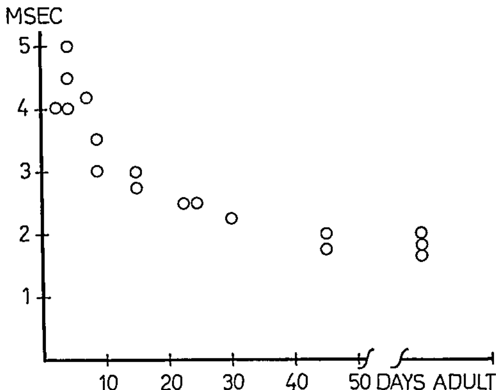


FIG 18 The minimum interval between two spikes recorded from single hair units in response to rapid strokes of a brush over the receptive field plotted against age The unit that gave the shortest interval (regardless of the number of spikes in the spike train) in each animal was selected

frequent rapid strokes of a brush A receptive field was stimulated manually in this way as frequently as possible during one minute The response was not apparently altered during the period of repeated stimulation the frequency and number of impulses were essentially unchanged both in cats and in kittens Thus, tested with this simple method the ability of the hair units to respond repeatedly with a train of impulses at the frequency characteristic for the particular age of the animal seemed to be the same in both adult cats and kittens but the period of repeated stimulation was probably too short to reveal any differences between immature and mature units

Receptive fields The hair units respond to movements of the hairs only within a circumscribed area the receptive field of the unit When the present study was started it was found that the data on the receptive fields of the hair units in adult cats were incomplete A comparison of

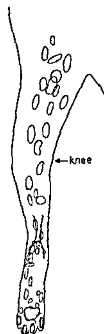


FIG 19 Diagram showing the shapes of the receptive fields of hair units

the size form and localization of such receptive fields was therefore undertaken in both adult cats and kittens Such a study is also of interest for the comparison between the receptive fields of peripheral and central neurons

The shape of the receptive fields of some adult hair units is illustrated in Fig 19 Out of 80 units that were studied in this way 83 7 % had approximately oval receptive fields usually with the long axis along the limb 12 5 % of the receptive fields were circular (10 % situated on the foot and distal part of the lower limb and the remaining 2 5 % more proximally on the limb) In a few cases (3 8 %) the shape was irregular Overlapping units were frequently found

In Fig 20 the surface area of the receptive fields of hair units in 72 adult (filled circles) and 36 non adult (open circles) (less than 30 days old) animals has been plotted against the actual position on the limb since all hindlimbs are not of equal length plotting against a unit length would be erroneous The sketch of the hindlimb serves as the abscissa against which the receptive areas have been plotted (actual longitudinal position) The range of the areas of the adult receptive fields is as seen in the Figure very great and exhibits a progressive increase in a proximal direction In the adult animals areas of greater than 150 mm were not observed on the foot and

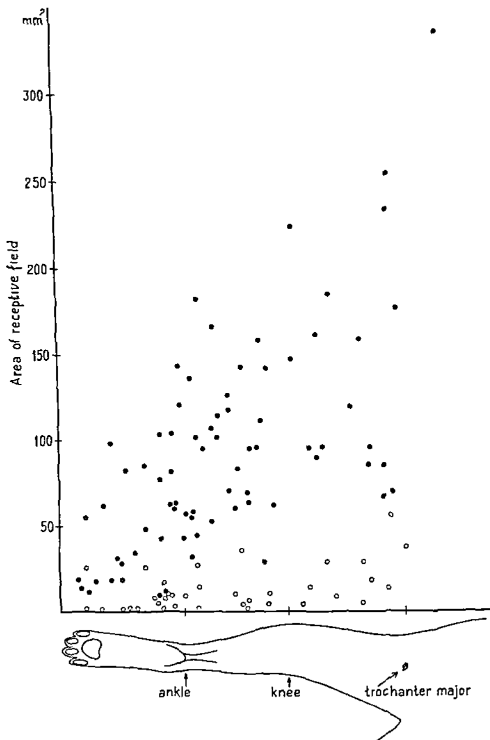


FIG 20 The surface area of the receptive fields of hair units plotted against actual longitudinal position on the limb The sketch of the hindlimb serves as abscissa Filled

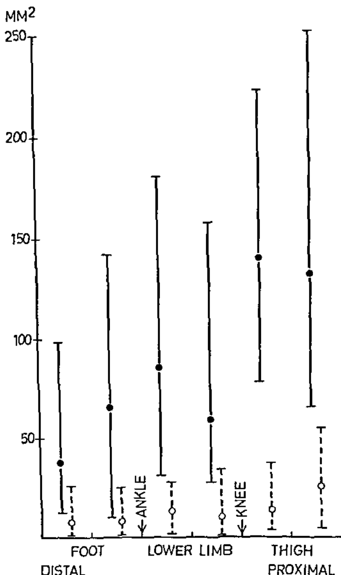


FIG 21 The average and range of the surface area of the receptive fields of hair units localized in six limb segments For further explanation see text Continuous line and filled circles adult units Broken lines and open circles non adult units (< 30 days)

there were no areas less than 50 mm on the thigh The non adult values were mainly in the low range and below the adult range

The foot lower limb and thigh were each divided transversely through their midpoints into proximal and distal parts Thus in all there were six

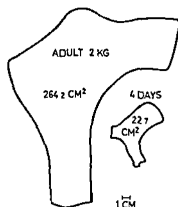


FIG 22 Total hindlimb skin area in a 4 days old kitten and an adult cat

limb segments The average and range of the receptive fields situated in each such segment was calculated and plotted in Fig 21 As can be seen the average area of the adult receptive fields was 37 5 mm² on the distal foot whereas the average area was 131 6 mm on the proximal thigh The ratio between the average area in the proximal thigh and the distal foot was thus 3 5 In the kittens the average area of the receptive fields was 7 0 mm on the distal foot and 25 5 mm on the proximal thigh

The skin area also increases during growth As shown in Fig 22, the total skin area of the hindlimb increases about tenfold between the age of 4 days and the adult stage

Because of the technical difficulties only a few single units could be completely analyzed in each young kitten Therefore the non adult material is smaller than that required for a strict statistical comparison with the adult material However large receptive fields were never seen in young kittens The receptive areas were small in the young kittens and indeed often too small to be accurately measured with the method used The shapes of the receptive fields in kittens appeared to be essentially the same as in adult cats but the proportion of round receptive fields might be somewhat greater than in the adult material The real occurrence of over lapping receptive fields in the young kittens was also difficult to establish Overlapping receptive fields were very seldom seen in young kittens but this may have been due to the small number of units studied in each animal

C THERMOSENSITIVITY OF AFFERENT UNITS

1 *Earlier observations*

The temperature sensitivity of afferent units in the hindlimb of adult cats has been studied by *Witt* and *Hensel* (1959) *Hunt* and *McIntyre* (1960 a)

and *Iggo* (1963 a) It has been found that many of the mechano receptors of the hindlimb skin are also sensitive to changes of the temperature, usually the units are excited by a sudden fall of the temperature and inhibited by sudden warming Some of these units have been shown to give a frequency maximum at a certain constant temperature level The temperature range associated with disharge is approximately 20—45°C

2 *Present observations*

The effect of sudden cooling was studied by recording from thin dorsal root filaments in kittens of different ages In the newborn animals sudden cooling usually gave rise to a discharge of relatively short duration This response could be produced in filaments that had no spontaneous activity before and after cooling but the fibres were sensitive also to mechanical stimuli Spontaneously firing units were also observed in the newborn kittens but were less frequent than in older kittens and adult cats Spontaneously firing units were not seen in recordings from the whole sural nerve in young kittens This is probably due to the shortcomings of the technique and the paucity of such units as compared with older kittens The spontaneously firing single units tested in newborn kittens usually responded on sudden cooling with a sudden frequency increase which then reverted to about the initial level Some of these units were tested also at different constant temperatures It was found that such units had frequency maxima at certain temperature levels as in adult cats Such temperature curves from

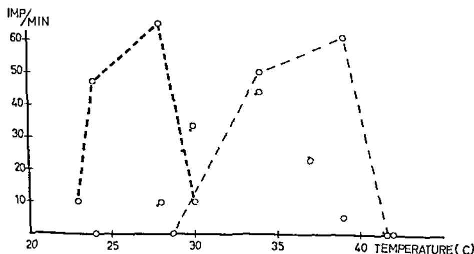


FIG 23 5 days old kitten Steady discharge frequency (imp/min) of three mechanosensitive afferent units plotted against constant skin temperature (°C)

a 5 day old kitten are shown in Fig 23 These units were also sensitive to mechanical stimulation of the skin

In the newborn kittens there are thus, units that respond to sudden cooling and to different constant temperature levels The findings obtained in older kittens do not differ essentially from those of the young kittens except for higher frequencies and the more frequent occurrence of spontaneously firing units

D TOUCH UNITS IN HAIRY SKIN

1 *Earlier observations on adult units*

Slowly adapting mechano sensitive units have been described in rabbits by *Frankenhauser* (1949) in the toad and cat by *Maruhashi Mizuguchi* and *Tasaki* (1952) in the cat by *Zotterman* (1939) *Witt* and *Hensel* (1959), *Hunt* and *McIntyre* (1960 a) *Wall* (1960) *Iggo* (1963 a) and *Tapper* (1964) in cats and monkeys by *Werner* and *Mountcastle* (1965) in monkeys and baboons by *Iggo* (1963 b) and in man by *Hensel* and *Boman* (1961)

A general property of the slowly adapting mechano sensitive units (Type I *Iggo* 1966) of the adult cat is that they respond to a maintained mechanical stimulus with an initially rapid discharge at the onset of the stimulus and then after a few seconds the rate of discharge slowly declines but persists as long as the stimulus is maintained The first phase appears as a peak and the latter as a plateau on the frequency curve

As regards the maximal frequency of the initial peak of the discharge demonstrable in adult cats it is not unusual to observe frequencies of about 400 imp/sec or more (*Hunt* and *McIntyre* 1960 *Tapper* 1964) *Iggo (1963 a)* has reported 1100 imp/sec The persistence of the discharge on sustained mechanical deformation of the skin that is the total duration of the discharge has generally been found to equal the length of time for which the stimulus is maintained Few figures are reported but it is clear that the activity always continues for several minutes afterwards (5 min or more *Iggo* 1966)

2 *Present observations*

In the present study it was found that touch units in newborn kittens do not behave in the same way as in the adult cats The maximal frequency that could be produced by physiological stimuli during the initial peak was lower than in adult animals No pronounced adaptation plateau followed after the initial peak Instead the discharge frequency of the units usually dropped to zero very quickly Fig 24 shows parts of three records which were obtained (A) in a newborn (B) a 3 weeks old kitten and (C)

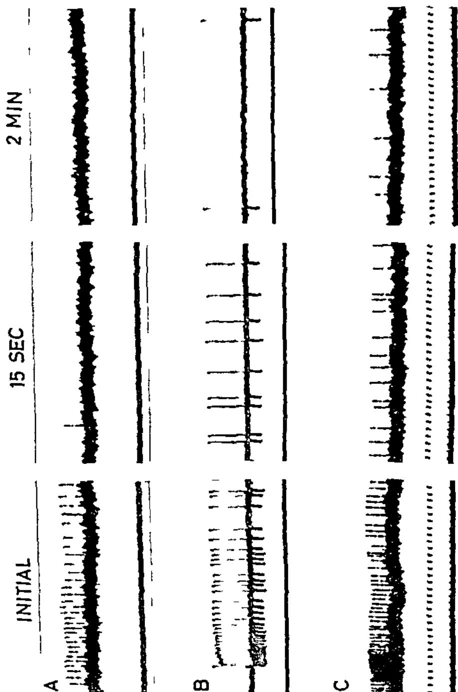


FIG 24 Recordings from single touch units in (A) newborn (B) 3 weeks old and (C) 6 weeks old kittens Sustained constant strong mechanical stimulation The figures indicate time after onset of stimulation Note low frequency and short duration in A moderate frequency but long duration in B and high frequency and long duration in C Note reduced spike amplitude at maximal frequency (Spikes retouched) Time 10 msec

Fig 25

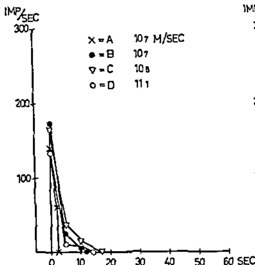
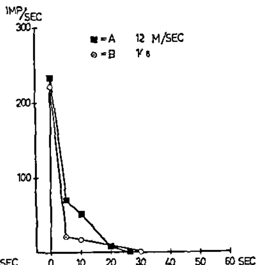


Fig 26



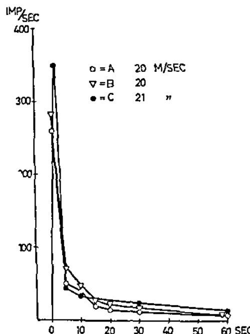


Fig. 27

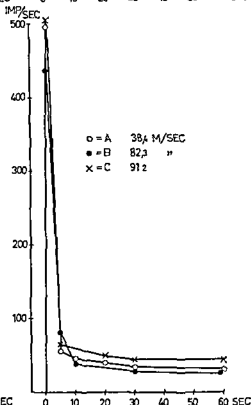


Fig 28

FIGS 25—28 Graphs showing the postnatal changes in the discharge pattern of single touch units in response to the same strong sustained constant pressure Impulse frequency plotted against time The unit that gave the highest initial peak frequency in each animal was selected The receptive fields of the units were situated in the sural skin area The conduction velocity of the fastest conducting fibres in n suralis is indicated in the figures Note the increase in peak frequency and discharge duration with

a 6 weeks old kitten Strong constant stimuli were used As can be seen the initial peak frequency was low in A, higher in B and highest in C After 15 sec the frequency of the unit shown in A dropped to zero whereas the discharge of the other two units continued after 2 min The adaptation frequency was higher in C than in B In the records it can also be seen that the spike amplitude was reduced at the maximal frequency

To further illustrate the postnatal changes of the discharge pattern the frequency of the most efficient (i e those giving the highest peak frequency on strong sustained constant pressure by means of the same clip) touch units from a series of animals of different ages have been plotted against time In Fig 25 four frequency curves of touch units from young kittens have been plotted One unit (A) from a kitten with a conduction velocity of 10 7 m/sec in the sural nerve gave an initial peak frequency of 140 imp/sec The frequency dropped to zero after 3 sec In another unit (B) from a kitten with the same conduction velocity the peak was 175 imp/sec and the frequency dropped to zero after 12 sec The third curve (C) shows the response of a unit in a kitten with a conduction velocity of 10 8 m/sec the peak frequency was 170 imp/sec and the duration of the discharge was 17 sec In one case (D) the peak frequency was 170 imp/sec and the duration of the response 14 sec, with a conduction velocity in the sural nerve of 11 1 m/sec

In slightly more mature kittens the units had higher peak frequencies and the frequency dropped to zero after a somewhat longer period of time In Fig 26 a curve (A) is shown from a kitten with a conduction velocity of 12 m/sec in the sural nerve the peak frequency was 235 imp/sec and the duration 26 sec Another unit (B) had a peak frequency of 222 imp/sec which dropped to zero in 30 sec (conduction velocity 14 8 m/sec)

The curves of Fig 27 were taken from kittens with conduction velocities of about 20 m/sec in the sural nerve It can be seen that the type of discharge is changed There is now a plateau of an adapted frequency which is more pronounced than in the early ages The frequency however was low as compared with older kittens and adult cats The discharge persisted for a longer period of time than in kittens with conduction rates below 20 m/sec in the sural nerve A and B illustrate the findings in two kittens with conduction velocities of 20 m/sec in the sural nerve The initial peak frequencies were 260 and 280 imp/sec respectively The units were still firing with frequencies just below 10 imp/sec 1 min after the start of stimulation The unit C was found in a kitten with a conduction velocity of 21 m/sec in the sural nerve It showed a maximal frequency of 350 imp/sec The adapted frequency was 12 imp/sec even 1 min after the start of the stimulation

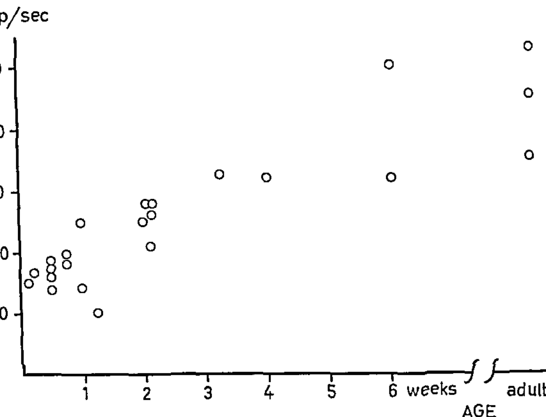


FIG 29 Maximal initial peak frequency of single touch units in response to the same constant strong sustained pressure plotted against age The unit that gave the highest frequency in each animal was selected

A unit found in a 45 day old kitten (conduction velocity 38 4 m/sec) is shown in Fig 28 (A) The peak frequency was 500 imp/sec and the plateau frequency was 25 imp/sec after 1 min Some adult units are also shown the configuration and frequency values of the curve (B C) are similar to those obtained in the 45 days old kitten

The findings with regard to the maximal initial peak frequency and discharge duration (adaptation) can be summarized as follows With a constant strong mechanical stimulation the maximal initial peak frequency of touch units is low in newborn kittens and increases with increasing age up to about 45 days This is illustrated in Fig 29 which shows peak frequencies in relation to age As can be seen there was a steady shift of the range towards higher values up to 6 weeks when adult values were attained This is consistent with the results obtained from hair units

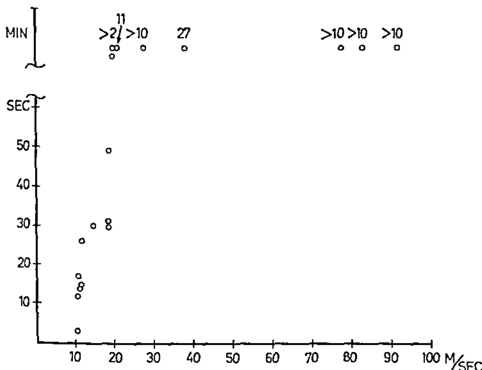


FIG 30 The duration of the discharge of single touch units in response to the same constant strong sustained pressure plotted against conduction velocity of the fastest conducting fibres in n suralis The receptive fields were localized in the sural skin area The unit that gave the highest initial peak frequency in each animal was selected

The minimum interval between 2 spikes obtained by the same stimulation was also determined It was found that the minimum interval was large (5 msec) in young animals and decreased with increasing age to attain (2 msec or less) adult values at an age of about 6 weeks This is similar to the results obtained on hair units (Fig 18)

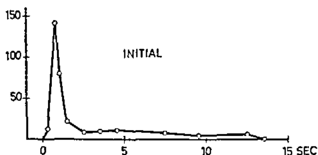
The duration of the discharge also increased with age The most immature units adapted rapidly to zero frequency with the persistent constant stimulus and were in this sense phasic At a certain stage of maturation the plateau -type of the frequency curves appeared This developmental phenomenon of the discharge duration is illustrated in Fig 30 The duration of the discharge (the time during which the unit fired on a strong constant maintained stimulus) has been plotted against conduction velocity in the sural nerve One striking feature of the graph is the abrupt increase of the discharge duration at a conduction velocity of 20 m/sec From short times of less than 50 sec at conduction velocities up to 19 7 m/sec the duration of

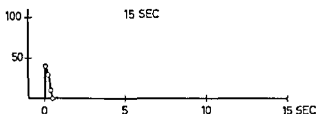
the activity increased to the order of several minutes This marked increase in the duration occurred at an age of 2—3 weeks, when, as shown in Chapter VI the conduction velocity of the sural nerve reaches 20 m/sec With a view to determining whether this phenomenon was correlated with the finding of higher maturation of the nerve fibres proximally than distally as described in Chapter V attempts were made to compare the properties of units with receptive fields in the proximal part of the limb with those having their receptive fields in the distal part However, because of the small number of completely analysed units in each individual animal no conclusions could be drawn

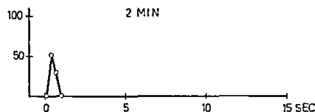
On the basis of the findings concerning postnatal functional changes described above a somewhat extended analysis was made in an attempt to further elucidate mechanisms limiting the activity of immature touch units

Reproducibility of response The first obvious question is How repro ducible is the response to a constant sustained stimulation? It was found that the frequency curves were fairly reproducible provided that the time interval between stimuli was sufficiently long and that the same stimulus intensity was used

This is shown in Fig 31 in a 4 days old kitten In response to the first sustained constant stimulation the unit adapted to zero in 13 5 sec The next (identical i e with the same clip) stimulus applied after 15 sec of recovery gave a response with a very low initial frequency and a duration of only 0 5 sec After a recovery period of 2 min the response was still not similar to the initial one When a recovery period of 10 min was allowed before application of the next stimulus the response was then similar to the initial one i e the peak frequency and the duration of the discharge were about the same as in the control response When a touch unit in a 45 days old kitten was induced to fire for 13 5 sec by the same stimulus and then again after a 15 sec rest period the second stimulus gave approximately the same response as the first Thus short stimuli can be repeated rather frequently in the more mature animals The question then arises as to what happens if such a mature unit is made to fire until it has adapted to zero When the same unit was stimulated until the frequency had dropped to zero (after 27 min) it was found that a 15 minute recovery period was required to obtain a response similar to the initial one Owing to the great technical difficulties only a small number of complete recovery curves were obtained and thus no definite conclusion can be drawn It is however quite obvious that the immature sense organs have to be tested very infrequently to allow any conclusions to be drawn with







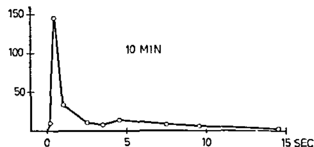


FIG 31 4 days old kitten The response of a single touch unit to the same constant sustained pressure stimuli repeated after recovery periods of 15 sec 2 min and 10 min Impulse frequency plotted against time

regard to their behaviour under different experimental conditions

When using strong stimuli, the discharge occasionally stopped abruptly and in this way the frequency curve ceased to be smooth Such units did not usually recover and were discarded because injury could not be excluded

Strong and weak stimulation The discharge of adult slowly adapting mechano sensitive units varies with the strength and type of stimulation The stronger the applied stimulation the higher are the initial peak and adaptation frequencies the duration of the discharge also increases (cf *Hunt and McIntyre* 1960 a *Iggo* 1963 a *Werner* and *Mountcastle* 1965)

During postnatal development the relationships between strong and weak stimuli and peak frequency were the same as in adult animals The duration of the response on the other hand became shorter when the stimulus strength was increased This is illustrated in Fig 32 in a 4 days old kitten First one strong clip was applied to the receptive field of a touch unit

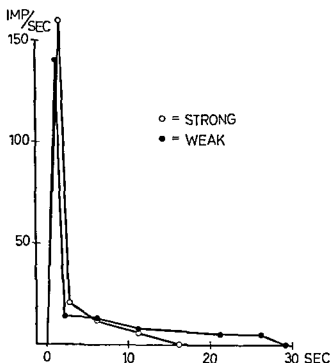


FIG 32 4 days old kitten The response of a single touch unit to strong (open circles) and weak (filled circles) constant sustained pressure stimulus Recovery period between the stimulations 15 min Impulse frequency plotted against time Note the short duration of the discharge in response to strong stimulation

After sufficient time for recovery (see above) a second weak clip was likewise applied It can be seen that strong stimulation gave the highest initial frequency but the shortest duration of the discharge

Very weak stimuli produced by finger pressure often caused a unit to fire for a considerable period of time While however use of a clip or a probe lying on the skin can be regarded as giving a constant stimulus level this is not so with finger pressure either directly or via an instrument Such a non-constant stimulus seemed to give longer duration of the discharge than a constant one in the sense defined here If the stimulus was intentionally made non-constant by means of alterations of the stimulus strength (rubbing) it could cause a unit to fire for a long period of time if the stimulus was not too strong

In immature touch units both the maximal firing frequency and duration of the discharge are less than in adult units With a strong stimulus while the maximal peak frequency is increased the duration of the response is shortened A weak stimulus has the opposite effect

Number of spikes From the above it is obvious that immature touch units produce overall a smaller total number of spikes in response to mechanical stimulation than adult units This raises the following question Is there a difference between the total number of spikes produced by weak and strong stimuli in the same immature unit?

Table X The total number of spikes produced by strong and weak constant stimulation

Age in days	Conduction velocity m/sec	Strength of constant stimulus	Max peak frequency imp./sec	Duration of discharge sec	Total number of spikes
4	11.6	strong	180	26	582
		weak	160	120	930
5	12.0	strong	200	38	516
		weak	180	130	1065

As can be seen in Table X the total number of spikes produced by a weak constant stimulus was 930 compared with 582 by a strong stimulus In an other experiment 1065 spikes were produced by a weak stimulus and 516 by a strong one The total number of impulses obtained from weak and strong stimuli thus differ the weak stimulus producing nearly twice as many action potentials as the strong stimulus

Electrical stimulation in the skin It has not been the aim of the present investigation to study the mechanisms responsible for the limitations

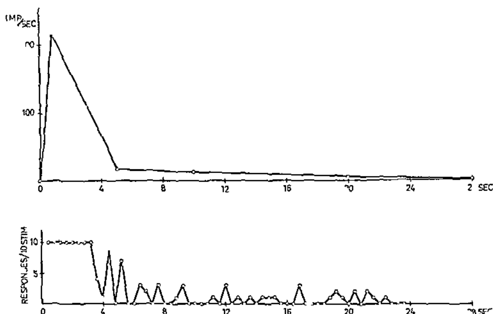


FIG 33 7 days old kitten The response of a single touch unit to a constant strong sustained pressure stimulus (above) and to electrical stimulation in the skin (25 imp/sec) at a stimulus strength just threshold for response (below) Recovery between the two types of stimulation 15 min Impulse frequency plotted against time (above) and responses per 10 stimuli plotted against time (below) Note increase of the threshold to electrical stimulation

of the activity of the sensory units in kittens Such a study requires other methods By using some simple experiments however indirect information can be obtained as to which part of the afferent unit may be responsible The afferent unit consists of a non nervous component in the skin the nerve terminations and the afferent nerve fibre In order to exclude the mechanical component of the stimulation of the afferent units electrical stimuli were delivered to the skin at the receptive field of the unit Using short trains of shocks of different frequencies the frequency level at which the one to-one stimulus response was broken was determined It was found that the one to one relation was maintained at higher frequencies in adult cats than in young kittens This indicates that the capacity of the immature fibre to fire with a high frequency is lower than in adult fibres This is consistent with the results obtained on hair units

Electrical stimulation (low frequency) for a long period of time was also tried (Fig 33) After mechanical stimulation and recovery the unit was electrically stimulated at a frequency of 25 imp/sec When the stimulus strength was supramaximal the units showed a one to one response for

a much longer period of time than when the discharge was induced by mechanical stimulation When however, the stimulus strength was adjusted to the response inducing threshold value the units showed a one to one response only initially The number of responses per 10 stimuli then progressively decreased in this case to zero and the latency between stimulus and response increased When the stimulus strength was increased when the response had reached zero the unit again showed a one to one response Different durations of the recovery periods were tested with the stimulus strength at just threshold values and unchanged throughout the test series It was found that stimuli free intervals (recovery periods) of considerable duration (5 min) were required to obtain two similar response series This shows that the increase of the threshold regardless of its cause lasts for a long time No definite differences were observed between kittens of different ages However a tendency for immature units to change these thresholds more quickly than more mature ones was observed

E ARTICULAR UNITS

Single units in dorsal roots In newborn kittens a few (3) units were identified as articular units localized to the knee joint These units responded with a discharge of very short duration on mechanical stimulation of the joint capsule and on passive movements None of these units fired spontaneously for more than a few seconds when the joint was in a resting position

Some further articular units were isolated in kittens 2—15 days old The properties of these units did not differ essentially from those described for the newborn kittens

In one 21 day old kitten an articular unit was found that gave a tonic (> 3 min) discharge when the knee joint was kept in a fixed position In kittens older than 21 days both this type of tonically discharging articular unit and rapidly adapting ones (duration of discharge about 1 sec) were found This is quite consistent with *Skoglund's* (1956) findings that in the adult cat both rapidly and slowly adapting receptors can be found in the joint capsule The slowly adapting sensory endings fired at a steady frequency for hours if after a period of adaptation from 0 5—1 min they did not reach zero This means that the above mentioned results i e the occurrence of only short duration responses up to an age of 21 days do not allow any conclusions as to whether or not development takes place from phasic to tonic responses The existence of two types of units and the fact that only a small number of articular units were studied makes it uncertain whether or not the responses of short duration were found by chance in kittens younger than 21 days In order to study further the

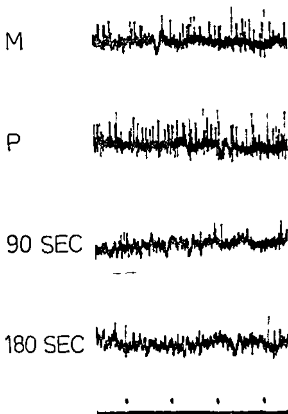


FIG 35 30 days old kitten Recording from the medial knee joint nerve The lower limb is passively moved from 135 —180° (maximal extension) and retained there in a fixed position M during movement P just after the fixed position is attained The figures indicate time after P Time 100 msec See text

be produced by movements of the joint or by steady pressure against the capsule and no steady discharge was observed with the joint in a fixed position In a 10 days old kitten responses with somewhat longer duration were obtained both by keeping the joint in a fixed position and by applying constant pressure to the joint capsule In the 30 days old kitten tonic responses which did not adapt to zero for a long period of time were obtained Single unit studies showed that the long duration responses could be obtained in kittens 3 weeks old At this age the fibre composition of the joint nerve (see Chapter V) corresponds to a conduction velocity of about 20 m/sec The results presented here are consistent with unpublished observations of *Skoglund* (1963)

The method of isolating single active fibres in the dorsal root quite naturally leads to a selection of the material in favour of the biggest fibres which appear to be the most mature ones functionally When comparisons have been made between units found in kittens of different ages, only the most efficient (with regard to frequency) units of each animal have been chosen In this way probably the most mature units in each stage have been compared though of course the material may not even be representative for the most mature units The material can therefore only be evaluated with regard to the developmental trends shown a strict quantitative analysis of the data might be misleading As regards the duration of the discharge the recordings from the whole nerves (large population of units) show a fairly good agreement with the single units studied Any discrepancies found can be attributed to the less satisfactory recording conditions when using undissected nerves or roots

It might be argued that the limited duration and frequency of the discharge seen in the early developmental stages is due to injury to the units But the reproducibility of the repeated discharges after a suitable recovery time and the smoothness of the frequency curves makes it unlikely that the limited action should be due to injury In response to strong stimulation the discharge sometimes stopped abruptly Such a frequency curve was not smooth and there was usually no recovery This was interpreted as due to injury and these units were discarded It is highly improbable however, that the articular units were blocked by injury when the joint was kept in a fixed normal position

The shapes of the receptive fields of the hair units presented here are essentially similar to those obtained by *Wall* (1960) and *Hunt* and *McIntyre* (1960 b) The range of the areas of the receptive fields found is consistent with the earlier observations of *Yamamoto* et al (1956) *Pulettt* (1959) and *Hunt* and *McIntyre* (1960 b) The present findings do not however confirm the findings of *Wall* (1960) that the largest area of units sensitive to light brushing of skin or hairs on the proximal hindlimb was only 18×10 mm (141 mm) and that the areas changed very little from the foot to the pelvic region In the present investigation hair units with larger areas than those reported by *Wall* were found on the proximal limb and it was found that the average area increased 3 5 times between the distal part of the foot and the proximal thigh The discrepancies between *Wall's* findings and those presented here may be due to the different methods used for classification and stimulation of the units

The areas of the receptive fields in young kittens were smaller than those

in adult cats This is what might be expected since the total skin area of the hindlimb increases 10 times between birth and the adult stage This figure is also similar to that found by *Boyd* (1935) for the skin surface increase in man According to *Szabo* (1958) all hair follicles are already present at birth The hair follicles must then spead out postnatally concomitantly with the increase in the total skin area If nervous contact has been established with the hair follicles the receptive areas of each hair unit must also increase This is supported by the finding that most of the receptive areas of young kittens are smaller than the smallest ones in adult cats Another possibility is that all hair follicles are not innervated at birth and that such contacts are established during postnatal development

Although the temperature sensitivity of myelinated mechano sensitive units in the hairy skin of cats is considered to be of minor importance for the sensation of temperature (*Hensel* 1966 a) it is interesting to note that the temperature sensitivity seen in young kittens was essentially the same as in the adult cats Whether or not it results in sensation cooling evokes an increased activity in the afferent nerves and thus changes in the environment are transmitted centrally in kittens If cooling gives rise to motor activity the kitten further has a system which enables it to react to a remote stimulus

From the firing frequencies of afferent units it can be concluded that the receptors subserving different modalities in the skin show the same general traits in their postnatal development Thus in the newborn stage they have low firing frequencies which gradually increase with increasing age to attain adult values some 40—50 days postnatally The fibre diameters however continue to increase after this age (Chapter V) but it is interesting to note that the density of mitochondria in the paranodal region attains adult values at about the same time (*Berthold* and *Skoglund* 1967)

All receptors studied adapt relatively rapidly in the newborn stage in response to constant strong stimuli For those having such properties in the adult stage the development does not of course give rise to any obvious changes Nor was it possible to investigate their properties other than with regard to firing frequently The adaptation of the cutaneous receptors developing into slowly adapting units in the real sense on the other hand has been analyzed in considerable detail

It was then found that the duration of the discharge in response to constant strong stimuli showed an abrupt change at 2—3 weeks postnatally For the most effective units in the sense defined earlier this change was correlated to the attainment of a conduction velocity of 20 m/sec

However before this stage of development was reached a strong constant stimulus always gave a short discharge with a high initial frequency and short

duration whereas a constant weak stimulus gave a lower initial frequency but a considerably longer duration of discharge This is in sharp contrast to the activity obtained from adult receptors where a strong constant stimulus gave a high initial frequency and a high adaptation frequency of long duration with a very large number of spikes In the immature units on the other hand the weak stimulus produced a greater total number of spikes than the strong stimulus The articular receptors too gave shorter responses when strong stimuli were used

The observation of an overall low frequency of immature receptors indicates that the recovery phase of the impulse mechanisms of immature units is not as fast as that of adult units The finding that the adaptation is faster after a high initial frequency of response points in the same direction with a slow recovery process the overall low ability of the fibre to produce spikes will be more quickly exhausted This is in good accordance with the observation of an increased relative refractory period found in immature fibres (*Skoglund* 1960 c) and is further supported by the observation made here that the spike amplitude decreases when the units fire at maximal frequencies in response to mechanical stimulation since this indicates that the spikes are in the relative refractory period of the preceeding spikes (*Iggo* 1963 a *Tapper* 1964) Low frequency seems to be characteristic of afferent units in the muscle joint and skin and the same has been found in spontaneously firing diencephalic neurones in young rabbits (*Hyvarinen* 1966) Thus it appears likely that the low frequency is characteristic not only of some immature peripheral afferent units but also of other immature neurones

The experiments in which electrical stimulation of the afferent units was used showed that the one to-one relation between stimulus and response was broken at a lower frequency in the immature units than in the more mature ones This is in good accordance with the longer absolute refractory period found in immature fibres by *Hursh* (1939 b) and *Skoglund* (1960 c) This would also partly explain the low frequencies obtained from immature afferent units on adequate stimulation since their maximal frequency will be limited by that of the fibre

In the present study an increase of the threshold and the latency was obtained during iterative electrical stimulation of touch units The effect usually had a duration of about 5 min after interruption of the stimulation With reservation for the shortcomings of the method the results might indicate that hyperpolarisation of the fibres is induced by iterative stimulation (cf *Ekholm* and *Skoglund* 1964 b)

The results of the present analysis of the postnatal functional changes of the exteroceptors in the skin and the proprioceptors in the joint are in good accordance with the observations made by *Skoglund* (1960 c 1963)

Weed (1917) studied reactions induced by skin stimuli in decerebrated kittens Wirthdrawal of the leg occurred on pinching the foot or foot pad, the youngest kittens being reported as the most active In older kittens greater excitation was required but the threshold was still low as compared to that of the adult cats As regards also other general reactions elicited from the skin *Weed* (1917) emphasized that kittens were all far more responsive to excitations used than are adult decerebrated cats'

Windle (1929) found that after decerebration 'pinching the foot pad in kittens resulted in homolateral drawing away of the foot and often the crossed extension followed A newborn kitten was very irritable to tactile stimuli whereas a 26 days old kitten was 'less irritable and required slightly greater stimuli to produce the same reaction *Windle* (1930) also investigated behavioral reactions in intact kittens and found that various stimuli cause avoiding responses, the head or the legs tend to move away from the source of the stimulus, and quick reactions that involve many groups of muscles occur when the stimuli are painful

Skoglund (1960 a) found that in decerebrated newborn kittens pinching one hindlimb was followed by withdrawal of that limb and in the contra lateral limb it evoked a series of movements of flexion and extension ending in a contralateral flexor reflex Skin stimuli caused movements in all four legs but the reactions to skin stimuli in the decerebrated preparations were successively reduced during the postnatal development

The occurrence of rhythmic movements in the limbs in response to skin stimulation has been reported in the prenatal (*Graham Brown* 1915, *Windle* and *Griffin* 1931) and the postnatal period (*Weed* 1917 *Windle* 1929 *Skoglund* 1960 a) Using electromyography *Glebovsky* (1956) recorded activity in knee antagonist muscles of kittens in response to stimulation of the sole of the foot He claimed that the antagonist activity was both alternating and simultaneous

In curarized decerebrated or spinal kittens *Malcolm* (1953 1955) found the first signs of inhibition at 3—5 days of age He reported that this inhibitory activity came from the skin but no information was given about the type of stimulation used i e whether there was mechanical or electrical stimulation of the skin or electrical stimulation of a skin nerve

The investigations made (*Weed* 1917 *Windle* 1929 *Skoglund* 1960 a) on the reactions of decerebrate kittens in response to skin stimuli show that such stimuli have a great effect on the motor system whereas the proprio ceptive reflexes are less effective (*Skoglund* 1960 a) This dominance of the reflex responses to excitation of cutaneous sense organs is present in spite of the immature behaviour found in these sense organs as shown in Chapter VII

Thus it remains to consider the second main question posed (Chapter I) of whether the cutaneous sense organs owe their greater influence on motor activity during development to the state of central excitability and organization prevailing at that particular stage of postnatal development

In order to analyze the postnatal changes in the cutaneous reflexes of the kittens the following were studied a) facilitation and inhibition of monosynaptic reflexes by electrical stimulation of a cutaneous nerve and b) the effects that can be induced from the excitatory and inhibitory skin areas for hindlimb muscles using adequate stimulation of the skin as described by *Hagbarth* (1952)

No information about the excitatory and inhibitory skin areas for hindlimb muscles in animals in the pre or post natal period has been found in the literature Some of the results presented here were published in a preliminary report (*Ekholm* 1967)

The question considered in the present work is whether there are differences between young kittens and adult cats with regard to 1) excitability changes of motoneurone pools following a) electrical stimulation (single shocks and iterative stimulation) of a cutaneous nerve and b) mechanical stimulation of the skin 2) the overall response in the muscles induced by mechanical stimulation of the skin and 3) the excitatory and inhibitory skin areas for flexor and extensor muscles

These results can be compared with the state in adult cats since much is known about these mechanisms in the latter (*Lloyd* 1943 *Bernhard* 1947, *Hagbarth* and *Naess* 1950 *Hagbarth* 1952 *Megirian* 1962 *Eccles* and *Lundberg* 1959 a b *Holmqvist* and *Lundberg* 1961 *Lundberg* 1962) References to these investigations are made in connection with the experimental results obtained here in kittens

Material and Methods

Successful experiments were performed in 56 kittens ranging in age from 1—90 days

I Experiments with electrical stimulation of n suralis

These experiments were performed on animals anaesthetized with intra peritoneal pentobarbital (Nembutal®) the anaesthesia being maintained at as light a level as possible Generally the dose was 25—35 mg/kg body weight Lumbosacral laminectomy was performed and the ventral roots L7 and S1 were dissected free and cut near their exits from the dural sac In the

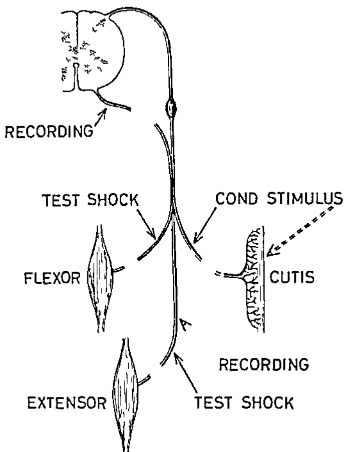


FIG 36 Diagram showing the experimental arrangement when the monosynaptic method was used

popliteal fossa n suralis was cut and dissected free proximally as far as possible One extensor and one flexor muscle nerve were chosen 1) The nerve to the gastrocnemius muscles (both medial and lateral which also includes fibres to the soleus muscle) and 2) the nerve to the biceps semitendinosus muscle These nerves were cut as far distally as possible and dissected free proximally All other hindlimb nerves which were not being studied were cut

For the description of temperature control and protection of exposed tissue see Chapter III and of fixation of the animals Chapter VII

The experimental arrangements are illustrated in Fig 36 The test stimulus was applied either to the flexor or the extensor muscle nerves The conditioning stimulus was applied to the ipsilateral n suralis and the

recording was made from the ventral root (L7 or S1) The conditioning single shocks preceded the test shocks by 1—700 msec

Stimulation The conditioning stimulus on n suralis was a square wave of 0 3 msec duration which could be delivered either singly or as a tetanus The stimulus strength was usually supramaximal, this was checked by recording directly from the nerve The test shocks were also square waves of 0 3 msec duration applied to one of the muscle nerves the stimulus strength was usually supramaximal but was sometimes submaximal for the monosynaptic reflex The submaximal stimuli gave very unstable test potentials Two stimulators were used The shocks were triggered by delay circuits To minimize the risk of cross stimulation the shocks were delivered through one to one transformers The sweep intervals were regular and varied between 1 and 3 5 sec the longest intervals being used for the youngest kittens Ag AgCl hooks were used as stimulating electrodes the interpolar distance was generally 2—3 mm For details about the method of recording see Chapter III

In the experiments with iterative stimulation of n suralis the test reflexes were elicited at regular intervals (usually 2—3 sec in the small kittens and somewhat less in the older ones) and the tetanisation was initiated and interrupted by hand without change of the sweep intervals Thus the first observation of the effect of the tetanisation on the monosynaptic potential was made on the first sweep after the start of the tetanisation The period from the start of the tetanisation to the following first sweep varied within the limits of the sweep intervals used and the events during this early period were not studied in these experiments

II Experiments with mechanical stimulation of the skin

A tracheal canula was inserted under ether anaesthesia and both carotid arteries were ligated The animals were decerebrated by a section through the intercollicular region The section was made in such a way that the ant sup cerebellar artery was not damaged (see *Skoglund* 1960 a) One series of kittens were in addition to decerebration spinalized by a transection of the spinal cord at the level Th12 One hour was allowed for the ether effect to disappear

A Monosynaptic test reflexes The monosynaptic test reflexes recorded in this series of experiments were elicited from 1) the nerve to the medial and lateral gastrocnemius muscles which also contains fibres to the soleus muscle (as ankle extensor) and 2) the nerve to the anterior tibial muscle or the deep peroneal nerve (as ankle flexor) These muscle nerves were cut near the muscles and dissected free proximally in the popliteal fossa The nerves

to the hamstring muscles were cut The tibial nerve was usually cut but *since it contains cutaneous afferents from the foot and toes* it was sometimes left uncut when the effects of cutaneous stimulation applied to the distal parts of the planta pedis and digits were being studied

It was technically very difficult to fix the young kittens satisfactorily when experiments of this type were performed in unanaesthetized decerebrate and/or spinal kittens When the skin was stimulated mass reflexes in *the whole kitten* were often evoked These movements were strong enough to damage any tissue which had been used for fixation Because of incomplete calcification in the young animals no suitable osseous structures were available for fixation Denervation could not be made so complete as to exclude all movements as for example in the axial muscles of the trunk Curarized *animals were not used the main reason being that possible central effects* of this drug in young kittens are not known However out of the large number of animals in which these experiments were attempted some were successful and [illegible] results will be reported here Fixation was performed in two ways Rigid fixation was tried, where the animal was nailed to a cork support A more flexible type of fixation was also tried to avoid damage [illegible] the tissue during mass reflexes Whether one of the methods was superior to the other was never established both are rather *unsatisfactory*

The test potentials were usually recorded from a ventral root (Fig 36) but in order to obviate some of the fixation difficulties a somewhat modified experiment was performed The monosynaptic potential was recorded from the same nerve to which *the test shocks were applied (Fig 36) (see Wilson* 1962) The stimulating electrodes were situated more distally on the nerve than the recording electrode This reduced the fixation problem because small movements in parts of the body other than the hindlimb under study did not essentially interfere with the recording The hindlimb being studied on the other hand was rigidly fixed by nailing it to a cork support

For further details of the method see also Chapters III and VII Mechanical stimulation of the skin was performed by pinching with the fingers or forceps or by touching the skin with a probe or a finger (see Chapter VII) The quality of the stimulus varied from a light touch to stimuli which were nociceptive in character

The sweep intervals were 2 5—3 5 sec in the young kittens which was usually enough to get a fairly stable monosynaptic reflex although the potentials were not maximal if elicited so often (*Skoglund* 1960 d) In the older kittens somewhat shorter sweep intervals could be used First a series of fairly stable control test reflexes was established then with the same

sweep conditions the skin was mechanically stimulated for some time and then a new series of controls was recorded

B EMG recordings Reflex responses were recorded electromyographically (EMG) by coaxial electrodes inserted into the muscles through the skin The position of the tip was controlled by palpation and by the movements of the electrode induced by passive movements of the muscles Four muscle groups were chosen for EMG recordings 1) Triceps surae as an ankle extensor (gastrocnemius med and lat soleus and plantaris) 2) anterior tibial muscle (and other pretibial flexor muscles) as an ankle flexor 3) biceps semitendinosus as a knee flexor, 4) quadriceps as a knee extensor

Kittens were fixed in the prone position by nails passing through the bones of the limb into a cork support To check whether any nociceptive stimuli from the nails themselves essentially influenced the reflex pattern some animals were mounted by tape which should not be expected to cause any painful stimuli No differences were seen in the experimental results The results reported have been obtained only from kittens which were in a good condition after the operation

The activity recorded from the muscles was fed through an amplification system to a loudspeaker and to an oscilloscope which could be photographed (see Chapter III) In order to exclude that the recorded activity arose from adjacent muscles and not from the active muscle into which the electrode was inserted the presence of spike potentials in the appropriate muscle was always checked When mapping the excitatory and inhibitory areas attention was paid only to the response that followed immediately after the stimulus Usually only one channel was available for recording but sometimes two EMG recordings could be made simultaneously The occurrence of inhibition was tested against a background activity set up by stimulation of an excitatory area The tail was usually used for the excitation of the extensors and the foot pad for the excitation of the flexors (*Weed* 1917 *Skoglund* 1960 a)

Results

A THE EFFECT OF SINGLE SHOCK STIMULATION OF A CUTANEOUS NERVE ON IPSILATERAL MONOSYNAPTIC TEST REFLEXES

1 *Earlier observations on adult cats*

Following a conditioning shock to n suralis there is generally an early inhibition and a late facilitation of the ankle extensor motoneurones in adult cats (*Bernhard* 1947 *Hagbarth* and *Naess* 1950 *Hagbarth* 1952)

Administration of Dial® eliminates the late facilitation and prolongs the early inhibition (*Hagbarth* and *Naess* 1950)

An afferent volley from a cutaneous nerve generally causes an early facilitation of flexor motoneurones (*Lloyd* 1943, *Hagbarth* and *Naess* 1950, *Hagbarth* 1952) and often a late inhibition (*Hagbarth* 1952) This facilitation was not affected by administration of Dial® (*Hagbarth* and *Naess* 1950)

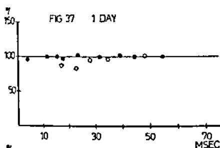
2 *Present observations*

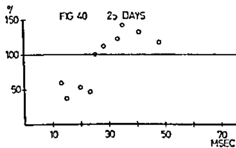
Monosynaptic reflexes from extensor and flexor muscle nerves were recorded from a ventral root in newborn kittens (*Skoglund* 1960 b) A typical finding from a newborn kitten is shown in Fig 37 which illustrates graphically the amplitude change of the test potentials following a single shock applied to n suralis Each value in the graph (and the following similar graphs) is the mean value of at least 5 and usually 10 consecutive monosynaptic potentials The control values are also the mean of an equal number of observations Supramaximal test stimuli have been used in all the experiments illustrated A very small (105 %) facilitation (control = 100 %) of the flexor test reflex was obtained after 8 msec but this value was within the range of the controls The extensor test potential showed a depression to 74 % of the control 6 msec after the conditioning stimulus and had returned to the control range after 35 msec

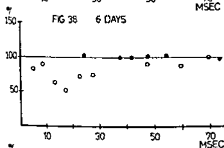
In kittens 5—6 days old (Fig 38) a facilitation of the flexor test response with a maximum of 120—135 % was obtained 10—15 msec after the conditioning stimulus In two cases out of five this changed later into a depression beginning 25 msec after the conditioning stimulus The extensor test response changed to an early depression sinking maximally to 53—60 % of the control value 13—18 msec after the conditioning stimulus The amplitude of the test response had returned to the control level after 70 msec in one case out of five in the others it remained depressed for longer periods up to 120 msec

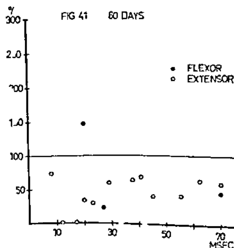
The facilitation of the flexor response in an 11 days old kitten (Fig 39) was 150 % and changed into a late depression The extensor test reflexes showed a depression with a maximum lowering to 42 % of the control about 10 msec after the conditioning stimulus

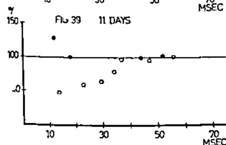
In Fig 40 the results from a 25 days old kitten are shown The early depression of the extensor test response can be seen The amplitude of the test potential decreased to 37 % of the control level with a minimum after 15 msec After 25 msec the reflex amplitude was back to 100 % and there was then a late facilitation amounting to 142 % with a maximum 35 msec after the conditioning stimulus This curve seems to have the same general shape with an early depression and a later facilitation as those found











FIGS 37–41 Graphs showing the postnatal development of the excitability changes in the motor nuclei of an extensor (gastrocnemius) and a flexor (biceps semitendinosus) following single supramaximal electrical shocks to n suralis The conditioned amplitudes of monosynaptic test reflexes recorded in the ventral root are expressed in per cent of the control amplitudes at each instance and plotted as a function of the time interval between conditioning and test stimuli The ages of the kittens are given in the figures Anaesthetic Pentobarbital For further explanation see text

by *Hagbarth* (1952) in adult cats although the size of the amplitude changes was not as great in the kittens

The results illustrated in Fig 41 were obtained in a 60 days old kitten The magnitude of the amplitude changes was greater than in any of the younger kittens The early depression of the extensor test reflex was complete (0 %) between 12 and 17 msec after the conditioning stimulus The facilitation of the flexor response was 2—3 times the control value The late depression of the flexor test reflex was also complete (0 %) 33—42 msec after the conditioning stimulus A late facilitation of the extensor response was not observed but there was a depression amounting to 59 % lasting about 100 msec

The results in this series of experiments can be summarized as follows following a single conditioning stimulus applied to the ipsilateral sural nerve in newborn kittens a very slight tendency to early facilitation of the flexor motoneurones was sometimes found A manifest early facilitation was present in 5—6 days old kittens and increased in amplitude during postnatal development In a 60 days old kitten the facilitation was of a magnitude similar to that found in adult flexor motoneurones The late depression of the flexor motoneurones was not observed in newborn kittens but at an age of 5—6 days a depression of relatively small amplitude was found This amplitude increased with increasing age and attained adult values in 60 days old kittens

Early inhibition of the extensors was present in newborn kittens and increased its magnitude during the postnatal development to attain adult values at the 60 day stage A late facilitation of the extensor motoneurones was found in a 25 days old kitten

B THE EFFECT OF ITERATIVE STIMULATION OF A CUTANEOUS NERVE ON MONOSYNAPTIC TEST REFLEXES IN KITTENS

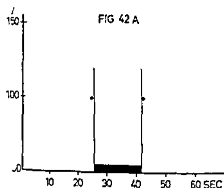
1 *Earlier observations on adult cats*

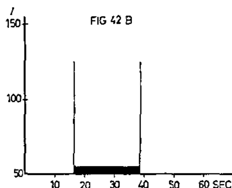
Iterative stimulation of n suralis causes a prolonged facilitation of both ankle flexors and extensors which can be eliminated by Dial® anaesthesia (*Hagbarth* and *Naess* 1950)

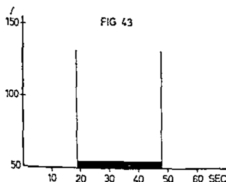
2 *Present observations*

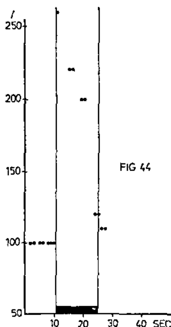
In the experiments made in young kittens although there was sometimes a slight change the amplitude of the monosynaptic test reflexes during iterative stimulation of the ipsilateral sural nerve usually remained unaltered Relatively low frequencies (80—90 imp/sec) were used in order to avoid blocking the immature fibres (cf Chapter VII and *Skoglund* 1960 e) If a change of the response occurred it was usually a slight increase to about 110 % The same effect was obtained for extensor as well as for flexor test potentials The facilitation of the monosynaptic potential often lasted only for the first one or two sweeps (sweep interval 2—3 sec)

In an 11 days old kitten the duration of the facilitation was longer as illustrated in Fig 42A which shows the amplitude of the monosynaptic test reflex elicited from the nerve to the biceps [illegible] plotted against time First 10 control test potentials we[illegible] and then the te[illegible] potentials during tetanisation of the ipsilater[illegible] The figure a typical result a relatively slight initial [illegible] that declines to









FIGS 42–44 Graphs showing the postnatal development of the excitability changes of the motor nuclei during iterative stimulation of the ipsilateral n suralis The conditioned amplitudes of monosynaptic test reflexes recorded in the ventral root are expressed in per cent of the control amplitudes and plotted against time A series of control test reflexes is shown before and after the test reflexes elicited during iterative stimulation of n suralis (indicated by the black area) Fig 42 11 days old kitten. A Flexor (biceps semitendinosus) tetanus frequency 80 imp/sec B Extensor (gastrocnemius) tetanus frequency 83 imp/ sec Fig 43 14 days old kitten Flexor (biceps semitendinosus) tetanus frequency 167 imp/sec Fig 44 60 days old kitten Extensor (gastrocnemius) tetanus frequency 182 imp/sec Anaesthetic Pentobarbital For further explanation see text

control level at the end of a tetanisation lasting 17 5 sec (tetanus frequency 80 imp/sec) After the tetanisation 10 control observations were made

In Fig 42B the extensor test potentials are plotted in the same way from the same kitten The slight facilitation declined during iterative stimulation (83 imp/sec for 22 5) but lasted in this case throughout the tetanisation

In 14 days old kitten (Fig 43) the initial facilitation of the flexor test response during tetanisation was somewhat greater (about 130 %) as compared with the findings in the youngest kittens described above The facilitation declined to control level during a 30 sec long tetanisation at a frequency of 167 imp/sec Similar effects were usually obtained on the extensor test reflex but in one experiment there was instead a slight depression

Fig 44 illustrates the findings in a similar experiment in a 60 days old kitten The effect of the extensor test reflex during iterative stimulation (frequency 182 imp/sec) for 15 sec was definitely different from that earlier described in young kittens The initial facilitation was 2—3 times the controls It declined slowly as in the youngest kittens but it had not returned to the control level after 15 sec The magnitude and time course of the effects during the iterative stimulation of the cutaneous nerve in a kitten of this age are of the same type as reported by *Hagbarth* and *Naess* (1950) for adult cats

The results obtained in the investigation of the extensor and flexor monosynaptic test reflexes during tetanisation of the ipsilateral sural nerve show that in the newborn stage there was usually no effect In some cases however a very slight effect usually facilitation occurred Later during development the facilitation of extensors and flexors was more pronounced At the age of 60 days the magnitude of the effect was similar to that in adult cats

C THE EFFECT OF MECHANICAL STIMULATION OF DIFFERENT SKIN AREAS ON MONOSYNAPTIC TEST REFLEXES

Monosynaptic test reflexes from the ankle flexors (n per prof) set up and recorded peripherally could sometimes be depressed by mechanical stimulation of the skin over the ipsilateral heel in decerebrate newborn kittens Generally however there was no effect at all unless the stimulus was so strong that mass reflexes were released Stimuli applied to the anterior part of the limb did not obviously change the test reflexes Enhancement of the flexor test potential was never observed The extensor test potential too was usually unaffected by skin stimuli

When the mass reflexes were present the monosynaptic test potential

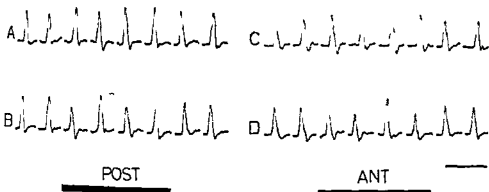


FIG 45 8 days old decerebrate kitten Extensor (gastrocnemius) monosynaptic test reflexes recorded from the ventral root S1 The tracings above the black areas show the test reflexes during pinching of the skin Control test reflexes are elicited before and after pinching post = pinching of posterior lower limb (heel) ant = pinching of anterior lower limb Time one msec Interval between test potentials 2 5 sec Note the small magnitude of the amplitude changes the inconstancy of the type of changes (slight facilitation in A and C alternating facilitatory and inhibitory effects in B and D) during pinching Note also the slight facilitation of the extensor induced by pinching the anterior lower limb For further explanation see text

was reduced This is probably due to refractoriness in the motoneurones and thus this method is not very suitable for measuring excitability changes of the ventral horn cells during mass reflexes

In a 7 days old kitten moderate stimuli which did not release mass reflexes were applied to different parts of the hindlimb skin Enhancement of the ankle flexor test potential was never seen The response was unchanged slightly depressed or showed a tendency to alternate between the control and a slightly lower level during mechanical stimulation of the skin The site of stimulation on the skin appeared to have no effect in determining the type of response

The alternating type of response was more obvious in an 8 days old kitten in which the extensor test potential was recorded from the S1 ventral root A test procedure consisting of a pinch of the skin over the heel which is known to be a region of high sensitivity for the excitation of the ankle extensors was repeated 4 times During mechanical stimulation of the skin over the heel slight facilitation occurred (110—120 %) in two trials (Fig 45A) and in another two trials alternating excitatory and inhibitory effects (B) were recorded The alternating responses were also of small magnitude Stimulation of the skin over the gastrocnemius muscle another potent excitatory area for the ankle extensors gave also alternating effects

When the skin of the anterior aspect of the lower limb (which in adult cats is an inhibitory area for this muscle *Hagbarth* 1952) was pinched in

the same experiment a slight enhancement of the extensor test reflex (C) occurred in 2 trials Alternating facilitation and inhibition (D) were found in one trial

When the area known to have maximal sensitivity for inhibition of the ankle extensor muscles in young kittens, the distal planta pedis was pinched a clear depression of the extensor test reflex occurred

The experimental data obtained from the young decerebrate kittens may by summarized as follows The monosynaptic test potentials were found to be relatively unaffected by mechanical skin stimulation Usually the stimulation did not affect the test potentials, but when it did the changes were rather small Furthermore the changes recorded during stimulation of a particular region show wide variation For instance on one occasion alternating changes were obtained and on another slight facilitation was seen The ankle extensor motoneurones were, however clearly inhibited from the distal planta pedis and sometimes slightly facilitated from the heel or anterior lower limb in an 8 days old kitten The ankle flexor motoneurones were usually unaffected but sometimes slightly inhibited from the heel and sometimes they gave alternating responses regardless of the area stimulated

It can thus be concluded that there is a difference between the young kittens and adult cats with regard to both the magnitude and direction of the changes of the monosynaptic test reflexes in response to mechanical skin stimuli In adult cats *Hagbarth* (1952) found a marked (up to 2—3 times the control value) facilitation of the gastrocnemius test reflex on adequate stimulation of the posterior lower limb and heel In the present study with young kittens such stimuli gave only slight facilitation or alternating changes of small magnitude From the anterior part of the lower limb *Hagbarth* (1952) induced a clear inhibition of the gastrocnemius test reflex in adult cats whereas this area gave a slight facilitation or alternating effects in the young kittens used in the present study

Hagbarth reported that in adult cats the ankle flexor motoneurones were facilitated from the whole limb except for the posterior lower limb and heel From the latter region inhibition could be induced This was not observed in the young animals tested in the present study Enhancement of the flexor test reflex was never seen and only occasionally could slight inhibition be demonstrated from the heel in young kittens

For comparison with the young kittens the findings from an older kitten (30 days) are shown in Fig 46 The monosynaptic potential from the ankle extensor was clearly facilitated from the heel and posterior lower limb and inhibited from the anterior lower limb The ankle flexor test potential on the other hand, was inhibited from the heel and facilitated from the foot and anterior lower limb Thus in a 30 days old kitten the adult basic

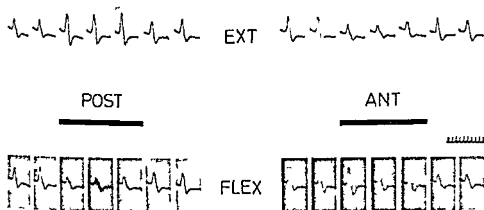


FIG 46 30 days old decerebrate kitten Extensor (gastrocnemius) and flexor (tibialis anterior) monosynaptic reflexes recorded peripherally on the muscle nerves The tracings above the black areas show the test reflexes during pinching of the skin post = posterior lower limb (heel) ant = anterior lower limb Control test reflexes are elicited before and after pinching Time one msec Interval between the test reflexes 2 sec Note the manifest facilitation and inhibition

pattern was present although the amplitude of the changes of the test reflexes was smaller than that found by *Hagbarth* (1952) in adult cats

Comment

According to *Granit* and *Job* (1952) monosynaptic testing gives a measure of the total number of motoneurones firing in response to each synchronous test shock i e the excitability level of those neurones that fire is sufficient for the transmission of a synchronous test shock *Granit* and *Job* (1952) designated this method a transmission test in distinction to electromyography which they named a discharge test The two methods were found to be at variance in some respects In monosynaptic testing thus facilitation and inhibition mean addition and subtraction respectively of motoneurones which fire in response to a test shock If the fringe is small or absent few or no fringe motoneurones are available for addition and therefore in the presence of a conditioning stimulus the control test stimulus has no additional neurones to mobilize and therefore the test response is not changed by conditioning Reduction of the test shock strength on the other hand may result in a diminished mobilisation which may be augmented by more excitable fringe neurones produced by a conditioning shock Being primarily a fringe test the monosynaptic test potential technique thus gives no information about the real discharge of the motoneurones The discharge in the muscle can be recorded however by means of EMG This

method was therefore used in order to get more information about the excitatory and inhibitory skin areas The EMG is often combined also with myography (*Hagbarth* 1952, *Skoglund* 1960 a) For the present study however, it was not considered feasible to dissect free the tendons in the small kittens without damaging the skin innervation and therefore myographic recordings were not attempted

D OBSERVATIONS OF REACTIONS TO SKIN STIMULATION IN KITTENS

Typical reactions elicited by skin stimulation in decerebrate kittens were reinvestigated The results obtained are essentially in accordance with earlier findings Comment is however made on a few points of interest

A normally active decerebrate kitten has a high responsiveness for reflex reactions elicited from the skin (*Weed* 1917 *Windle* 1929, *Skoglund* 1960 a) In a young kitten the elicitation of a response very often did not require a painful stimulus but only light touch In adult cats the evocation of a similar response regularly requires a painful stimulus The responsiveness decreases during a period of 2—3 weeks postnatally

The typical response to skin stimulation in a newborn kitten is a reaction which involves many muscle groups and joints (*Skoglund* 1960 a) Definitely localized responses are very seldom seen in the hindlimbs of newborn kittens but become more frequent with increasing age

If the distal planta pedis of the hindlimb in a young decerebrated kitten is pinched one or more of the following reactions occur

1) A simple withdrawal of the ipsilateral limb (*Weed* 1917 *Windle* 1929, *Skoglund* 1960 a)

2) The initial reaction is an extension which changes into an eventual flexor reflex The eventual flexor movement can also be preceded by alternating rhythmic flexions and extensions in one or several joints for varying periods of time after the stimulus has ceased (*Weed* 1917 *Skoglund* 1960 a)

3) In the contralateral limb there may be no response or the initial reaction may be a flexion (*Skoglund* 1960 a) and extension (*Windle* 1929) or alternating movements (*Skoglund* 1960 a) In the event of a reaction the final position may be either flexor (*Skoglund* 1960 a) or extensor Final flexion was usual in the youngest kittens and the extension in the older ones

4) Sometimes alternating movements may be present in all four limbs (*Weed* 1917 *Skoglund* 1960 a) and occasionally especially with a strong stimulus vigorous longlasting rhythmic activity spreads not only to limb

muscles but also to the trunk and neck (mass reflexes) This occurs more frequently in kittens than in adults

The plantar surface of the hindfoot is the most sensitive area for response but the above reactions can also be elicited from other parts of the limb Stimulation of the skin over the heel often elicited a movement which included a plantar flexion of the foot but was generally followed by a flexor reflex sometimes preceded by alternating movements At an age of about 3 weeks the reactions were adult like

E EXCITATORY AND INHIBITORY SKIN AREAS FOR EXTENSOR AND FLEXOR MUSCLES AS STUDIED WITH EMG

1 *Postnatal changes in the general response in the muscles*

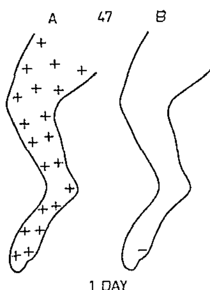
With EMG it is possible to determine which muscles are involved in the movements observed when mechanically stimulating the skin Thus the multi muscular responses involving several joints and the mass reflexes could be recorded in young animals The responsiveness to skin stimuli decreased and the occurrence of alternating responses diminished with increasing age

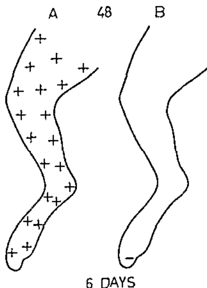
With increasing age stimulation of one skin locus activity gradually resulted in being limited to more well defined muscle groups Apparently the activity occurred more or less simultaneously in antagonistic muscle groups in response to stimulation of a skin area in newborn kittens (cf *Glebovsky* 1956) However further studies are necessary before any definite conclusions can be drawn since simultaneous recordings from antagonistic muscles must be made in a faster recording system

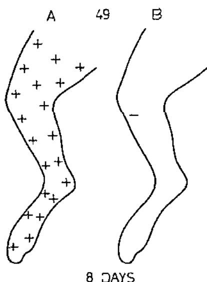
No differences in the response were observed when a short pinch and a sustained constant stimulus were compared in young kittens (cf Chapter VII about adaption of afferent units and earlier in this chapter about excitability changes during iterative stimulation of a cutaneous nerve) But if the stimulus was sustained and increased with time or of an alternating type (i e rubbing) or if frequent pinching were used the muscular response could be prolonged

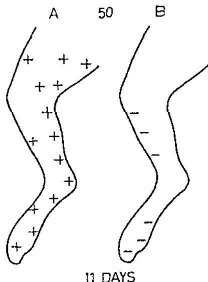
2 *Ankle extensors*

Activity in the ankle extensor muscles could be elicited by a moderate stimulus applied to any part of the ipsilateral hindlimb and tail in a newborn kitten The ipsilateral excitatory skin area diminished during the first 2—3 weeks postnatally to an area of the size and localization found in adult cats by *Hagbarth* (1952) viz the skin area covering the heel and triceps surae muscles on the posterior lower limb Inhibition in response

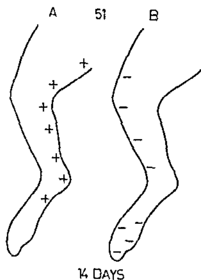


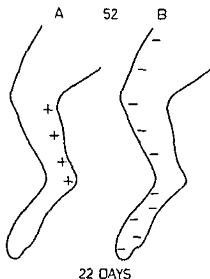


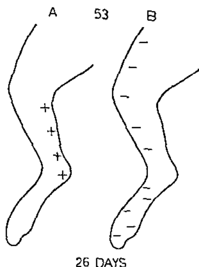




FIGS 47–53 Diagrams showing the postnatal changes in the excitatory (A) and inhibitory (B) skin areas for the ankle extensor muscles in decerebrate kittens (as studied by EMG) Age is indicated in the figures A plus sign indicates that excitation was induced from this site on the skin (by a pinch) A minus sign indicates that activity set up from an excitatory area was inhibited from this site on the skin Note the postnatal decrease of the excitatory areas and increase of the inhibitory areas For further explanation see text







to noxious stimuli was seldom found in newborn kittens It was observed however, when stimulating the distal part of the planta pedis that light touch often inhibited the ankle extensor whereas a stronger stimulus caused excitation The inhibitory area successively spread during the first 2—3 weeks postnatally until it was similar to that found in adult cats by *Hagbarth* (1952)

The results of a number of experiments are shown schematically in Figs 47—53 and illustrate the postnatal development of the excitatory and inhibitory skin areas for the nkle extensor muscles In the A figures, which show the excitatory ar i he plus sign means that muscular activity was elicited (if no spont ictivity was present) or increased (if spontaneous activity ( ) from the locus indicated Only one aspect of the limb is p the areas were generally symmetrically distributed on the lat al ral sides of the limb In the B figures, which show the inhib minus sign indicates that the muscular activity was inhibited of the skin

In Fig 47A th in a newborn kitten covers the whole ipsilateral limb T tivity induced continued for about 15 sec after a skin p exes were not elicited) The duration of the response wa stimulus was maintained at constant strength through Inhibition (Fig 47B) could be elicited (despite many tal part of the planta pedis only when very mild stim f the foot pad was lightly touched with a finger acti another source could be inhibited If the strength of th was increased a little the excitation was instead imm

The inhil ewborn animals (and at 6 and 8 days old as described below) ntical with the inhibition in older kittens The activity set up by stimulation of an excitatory area could be stopped by a suitable moderate stimulus of an inhibitory area but only very seldom did the muscular activity return spontaneously after removal of the inhibitory stimulus as is a common phenomenon in older kittens and adult cats

Fig 48 illustrates the results from a 6 days old kitten Muscular activity could be elicited from any part of the ipsilateral hindlimb skin In other respects also the findings were similar to those obtained in the newborn kitten Inhibition was produced by mild stimulation of the digits stronger stimuli increased the activity or evoked mass reflexes

In an 8 days old kitten (Fig 49) muscular activity could be elicited from almost all parts of the hindlimb skin although however, there was a difference in the stimulus intensity required for muscular response between the anterior and posterior aspects of the limb On the posterior side weak stimuli (touch) gave a response but on the anterior side somewhat stronger stimuli (noxious forceps) were necessary to evoke activity In this kitten inhibition could only be demonstrated from a small area on the anterior side just distal to the knee joint Often in kittens of this age, inhibition could also be produced from the distal part of the planta pedis

In an 11days old kitten (Fig 50) reflex activity was easily obtained by stimulation of the posterior aspects of the limb On the anterior side, however only some points gave response if strong pinches were used Inhibition could easily be produced from the distal planta pedis the digits and the anterior aspects of the lower limb

In a 14 days old kitten (Fig 51) the excitatory area was still smaller and covered only the posterior aspects of the limb The inhibitory area was larger than in the 11days old kitten i e the anterior sides of both the thigh and lower limb and the dorsal and plantar aspects of the foot and digits

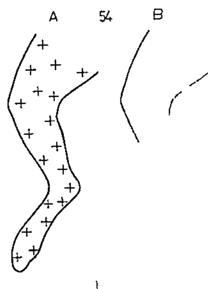
The findings illustrated in Figs 52 and 53 from 22 and 26 days old kittens respectively are essentially similar The excitatory area was small being limited to the skin area over the heel and triceps surae muscles on the posterior part of the lower limb Inhibition could be elicited not only from the distal part of the planta pedis and the digits but also from the anterior aspects of both the thigh and lower limb and the dorsal part of the foot

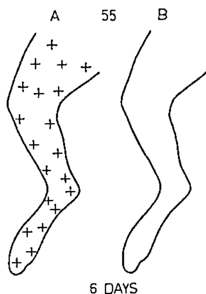
The findings described in the two kittens 22 and 26 days old are essentially in accordance with those of *Hagbarth* (1952) in adult cats

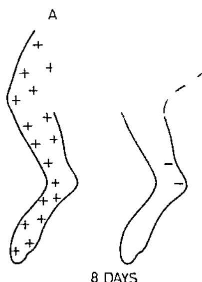
3 *Ankle flexors*

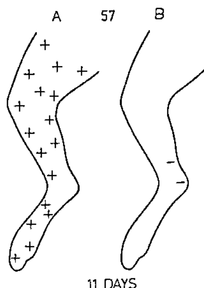
In Figs 54—60 the excitatory and inhibitory skin areas for the ankle flexor are illustrated in the same way as for the extensor in Figs 47—53 Since the trend of the po tnatal change of the excitatory and inhibitory skin areas for the ankle flexors is similar to that of the ankle extensors described above the skin areas attributed to the flexor are described here in a summarized form The excitatory area covered the whole limb in the newborn and 6-day old kittens (Fig 54A—55A) No inhibition could be demonstrated at this stage (Figs 54B—55B) In the 8 and 11 days old kittens (Fig 56—57) the excitatory area still covered almost the whole limb but a few points on the heel and posterior lower limb gave inhibition In kittens aged 14 days or older (Fig 58—60) excitation could not be elicited from the posterior lower limb and heel this area instead gave inhibition The size and localization of the excitatory and inhibitory areas for the ankle flexors are essentially similar to those found by *Hagbarth* (1952) in adult cats

For comparison typical findings in a newborn (A+B) and a 22 days old (C+D) kitten are illustrated in Fig 61 Stimulation of the skin over the heel elicited activity in the ankle extensor at both ages (A C) The stimulus applied to the anterior lower limb activated also the extensor muscles in the newborn animal (B) This skin area is also situated within the excitatory area of the ankle flexors (see Fig 54) This means that on activation of cutaneous sense organs in a certain locality an excitatory effect can



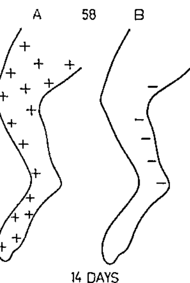


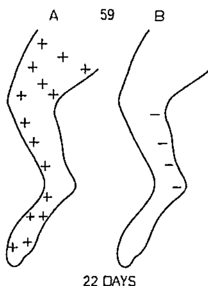


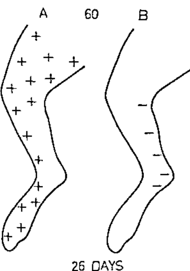


FIGS 54–60 Diagrams showing the postnatal changes in the excitatory (A) and inhibitory (B) skin areas for the ankle flexor muscles in decerebrate kittens (as studied by EMG) Other details as in Figs 47–53

be induced simultaneously in both the extensors and flexors of the ankle in immature kittens In the 22 days old kitten (D) on the other hand the extensor activity set up by stimulation of the tail was inhibited by pinching







the anterior lower limb This shows that an excitatory area in the newborn stage has become an inhibitory area in a more mature stage

It is thus obvious that the reflex effects from the skin change during postnatal development in such a way that the initially dominating excitatory action on both extensor and flexor muscles elicited from large cutaneous areas is gradually concealed and reverses into an inhibitory action elicitable

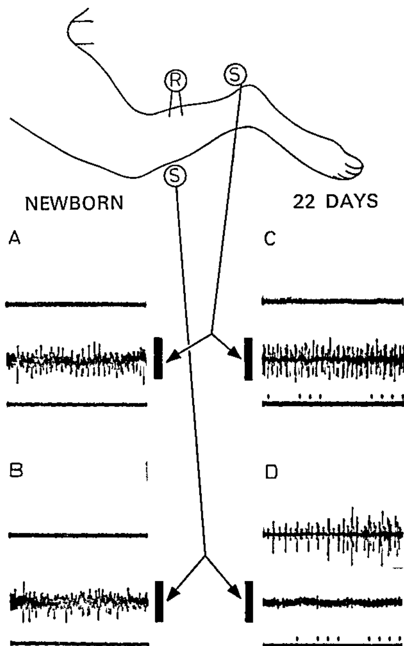


FIG 61 EMG recording (R) from ankle extensors A and B from a newborn C and D from a 22 days old kitten Upper tracing control Lower tracing mechanical stimulation (S) of the skin applied to the heel in A and C and to the anterior lower limb in B and D Time 100 msec Note that stimulation of the anterior lower limb activated the extensor muscles in the newborn kittens (B) but inhibited the activity in the 22 days old kitten

from progressively larger skin areas At the end of this development period the activity has also become reciprocal in the sense that the skin areas which are excitatory for the extensor muscle are inhibitory for the flexor and vice versa

4 *Spinalization*

In a number of kittens the experiments were performed both before and after spinalization and in these kittens very few differences were found up to an age of 2—3 weeks Up to this age no rigidity was obtained in the hindlimbs after decerebration (*Malcolm* 1955, *Skoglund* 1960 a) In kittens older than 2—3 weeks the extensor responses were of longer duration and easier to elicit before spinalization After spinalization the flexor responses were more easily elicited When the excitatory and inhibitory areas were mapped after the spinalization it was found that as a rule they had not changed to any great extent

5 *Knee extensors and flexors*

In order to compare the results obtained in the experiments on ankle muscles with the excitatory and inhibitory areas of the knee muscles the latter were also studied

In the same newborn kitten the responsiveness of the knee muscles was always lower than that of the ankle muscles This difference in responsiveness diminished progressively during the development until adult properties were attained at an age of about 2—3 weeks Thus in this sense the activity of the proximal muscles appears to be more mature than that of the distal ones This is an interesting observation in view of the finding that muscle spindles in proximal muscles have tonic discharges and gamma innervation earlier than those in distal muscles (*Skoglund* 1960 c)

The excitatory area of the knee extensors in newborn kittens usually covered the whole limb and gradually diminished up to an age of 14 days After this age excitation could usually only be obtained from the anterior thigh whereas the rest of the hindlimb skin area caused inhibition The latter pattern of the areas is similar to that found in adult cats (*Hagbarth* 1952 *Megirian* 1962)

In newborn kittens the excitatory knee flexor responses could be evoked from any part of the hindlimb and also from the anterior thigh Inhibition could not be produced from the anterior thigh In somewhat older (e g 7 days) kittens the responses in the knee flexors could be elicited from almost all parts of the limb On the anterior thigh however stronger stimuli had to be used for eliciting excitation than on the rest of the hindlimb

skin areas In a 22 days old kitten responses could be elicited from all skin areas except the anterior thigh In kittens of this age pinching the anterior thigh often inhibited the knee flexors This is consistent with the findings in adult cats (*Hagbarth* 1952 *Megirian* 1962) where facilitation of knee flexor motoneurones can be elicited from the whole hindlimb skin except the anterior thigh region from which inhibition is obtained

It can be concluded that there is a difference in responsiveness between the knee and ankle muscles in newborn kittens the former are less responsive than the latter (distal) muscles and in this sense the proximal muscles show a more mature behaviour The trend of the postnatal changes of the excitatory and inhibitory skin areas for the knee muscles is however essentially similar to that for the ankle muscles

6 *Responses to stimulation of the contralateral heel*

According to *Megirian* (1962) adequate stimuli applied to the contra lateral heel of adult cats generally facilitates the extensor and inhibits the flexor motoneurones In kittens from 1 to 8 days old activity was induced in both extensors and flexors in response to a pinch of the contra lateral heel From an age of 14 days however the activity generally only appeared in the extensor muscles in response to such a stimulus

Discussion

From the analysis of the firing characteristics of immature cutaneous sense organs (Chapter VII) it is obvious that the effectiveness of skin stimuli in producing motor responses in young kittens cannot be due to more efficient firing from these sense organs as compared with muscle receptors Thus the difference between young and adult animals must be attributed to differences in the central mechanisms of spinal reflexes However only the properties of myelinated cutaneous afferent units have been analyzed The myelinated fibres i e both group II (alpha and beta) and group III (delta) fibres as well as the unmyelinated fibres contribute to the reflex effects of the ipsilateral limb (*Lloyd* 1960) In view of the low firing efficiency of the unmyelinated afferent units in the adult state it seems unlikely that in the early stages of development they can compensate for the low firing ability of myelinated afferent units It cannot be excluded however that functional changes in the reflex effects of cutaneous afferents during development might be due in part to the development of unmyelinated afferents (see below)

Furthermore the local differences in sensitivity for the elicitation of

reflexes between different hindlimb skin areas (the distal planta pedis is the most effective area for the elicitation of the flexor reflex in the newborn and young animal) cannot be explained by a difference in functional maturation of cutaneous sense organs, for two reasons Firstly the firing properties of the immature cutaneous afferent units depend on the nerve fibre size (Chapter VII) and secondly if anything the nerve fibres of this sensitive reflexogenous area are less developed than the more proximal areas (Chapter V) It is more likely that the explanation lies in the high innervation density of the region (the plantar nerves contain a very large amount of fibres in relation to the size of the surface area which they innervate) and/or the central organisation of these afferents

The animals used for the experiments with conditioning by electrical stimulation of a cutaneous nerve were anaesthetized with pentobarbital because of the unsatisfactory conditions when such experiments are performed on unanaesthetized decerebrate kittens (see Methods) As shown by *Hagbarth* and *Naess* (1952) a similar drug Dial® did not affect the facilitation of flexors and the inhibition of the extensors was somewhat prolonged The late facilitation of extensors and the facilitation of extensors and flexors in response to iterative stimulation of a cutaneous nerve however were eliminated by this drug With the light Nembutal® anaesthesia used in the present investigation however both late extensor facilitation and facilitation in response to iterative stimulation of a cutaneous nerve could be demonstrated in older kittens This indicates that Nembutal® in the dosage used is not as depressant as Dial® It seems fairly justifiable to compare the results obtained in different animals anaesthetized in the same way although changes in the action of the drug during postnatal development cannot of course be excluded However it seems highly unlikely that the absent facilitation in newborn kittens can be explained by such an effect since the results were quite in accordance with those obtained in decerebrate kittens In the experiments where the test potential was recorded peripherally an antidromic effect must be considered Since *Skoglund* (1960 b) showed that such an effect is smaller in newborn kittens than in adult cats and that it lasts for about 100 msec it is not likely that it interferes much with the present results *Wilson* (1962) also used the same technique successfully in kittens

Excitability changes in the motoneurone pools were studied by means of monosynaptic potentials and conditioning by single shocks applied to a skin nerve A clear facilitation of the flexor motoneurones was not obtained until 5—6 days after birth but there was a small inhibition of the extensors in newborn kittens To ascertain whether the facilitation effect is absent in the newborn stage because a single conditioning volley in the skin

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The reflex patterns in the decerebrate animal were up to two or three weeks after birth essentially unchanged by spinalization, a result also obtained by *Malcolm* (1955) and *Skoglund* (1960 a) This means that the activity of supraspinal structures does not favour the hindlimb extensors in the flexor extensor balance until some time after birth Later, there is a postnatal change of the supraspinal control of the spinal reflexes that must constitute one factor responsible for the excitability changes on the spinal level

However influences on the motoneurones from other sources also change *Skoglund* (1960 a) showed that in decerebrate kittens the tonic stretch reflex was never obtained in the distal hindlimb muscles until some time after birth This means in sharp contrast to the conditions an adult cats, that a prolonged excitation of the motoneurones is not produced by activation of the sense organs in the muscle When a muscle contracts the contraction will only be inhibited by its own Golgi tendon organs causing autogenous inhibition since the muscle spindles lacking gamma support will cease firing in the young kittens (*Skoglund* 1960 c) The discharge from the Golgi tendon organs, though short and phasic may cause facilitation of the antagonist (*Granit* 1950 1955) which may in part be the cause of the alternating movements Recurrent inhibition may also contribute to this phenomenon The excitatory afferent input from the skin is unaffected by such mechanisms therefore it is more effective in activating motor performances in newborn kittens than the proprioceptors of the muscles Thus during postnatal development the source of the most effective excitation of the motoneurones is shifted from exteroceptors in the skin to proprioceptors in the muscles This postnatal change is further evidenced by the findings that proximal muscles are more mature than distal muscles with regard to responsiveness to skin stimuli and as found by *Skoglund* (1960 c) the proximal muscles have tonic discharges from muscle sense organs and gamma innervation earlier in development than distal ones Thus in spite of an increasing afferent inflow from the skin (Chapter VII) the most effective field for inducing motor activity is shifted from the cutaneous sense organs to the muscle receptors and this shift starts before the skin receptors have acquired adult properties but simultaneously with the appearance of tonic discharges and gamma innervation of the muscle spindles Furthermore in spite of the fact that the afferent input set up by electrical stimulation of skin nerves will become less synchronous, because of a wider size distribution among nerve fibres (Chapter V) whereby temporal summation is likely to decrease its effects on the monosynaptic potential will increase Hence the conclusion is drawn that the postnatal reduction of the effectiveness of skin stimuli in producing motor activity and the increased effects on monosynaptic potentials set up by stimulation of skin receptors or afferents cannot be due only if at all to changes in the afferent cutaneous

innervation but rather to changes in the excitability of spinal inter- and motoneurones. Even if the synaptic action of skin afferents increases this cannot explain the decrease in the motor effects obtained providing that the inhibitory action does not increase more than the excitatory.

It then remains to discuss the possibility that the postnatal change in the excitatory and inhibitory skin areas might be due to a change in the cutaneous innervation. It might be supposed that the cutaneous sense organs are so organized that in a specific skin area some have excitatory and some inhibitory connections with a specific motoneurone pool and that also in each skin area there is a certain ratio between the excitatory and the inhibitory receptors. Postnatal development leads to an overall reduction of the excitatory effects of skin stimuli and an increase of the inhibitory effects. This is also evidenced by the change in the excitatory and inhibitory skin areas for extensor and flexor motoneurones. The skin areas for excitation decrease and those for inhibition increase. In this process the earlier dominant excitatory effects are depressed but the excitatory connections are still there — the concealed reflexes in the sense of *Sherrington* (*Creed* et al. 1932) which can be unmasked under certain experimental conditions as for instance after spinalization (*Hagbarth* 1952, *Job* 1953, *Lundberg* 1962). In this respect the innervation remains unchanged. In view of the magnitude of the excitability changes of inter- and motoneurones it is not necessary to assume that a change in the ratio between excitatory and inhibitory receptors in a certain skin area has to occur in order to explain the postnatal change of the skin areas but it cannot of course be excluded. However it should be noticed that the number of myelinated nerve fibres in a cutaneous nerve continues to increase for a long time after the stage of development when the excitatory and inhibitory skin areas present in the adult stage have been established (2—3 weeks). Therefore no great change in the ratio between excitatory and inhibitory afferents takes place after this stage in spite of the considerable change in number of fibres. Consequently it appears less likely that such a change in the ratio between excitatory and inhibitory afferents should have taken place before an age of 2—3 weeks and then stopped. On the other hand a change in the synaptic action in favour of inhibitory afferents might contribute to the change in the effects obtained.

Summary

1) In order to analyze the postnatal changes in cutaneous reflexes of the hindlimb the following experiments were made: a) flexor and extensor monosynaptic test potentials were conditioned by either electrical stimulation (single shocks or tetanus) of an ipsilateral cutaneous nerve in kittens anaes-

thetized with pentobarbital or by adequate skin stimuli applied to different skin areas of decerebrate kittens and b) EMG was recorded and the excitatory and inhibitory skin areas for extensor and flexor muscles mapped in decerebrate kittens before and after spinalization

2) Following a single conditioning stimulus applied to the ipsilateral sural nerve a manifest facilitation of the flexor test potential was not observed until an age of 5—6 days postnatally but there was a small inhibition of extensors in newborn kittens The magnitude of the facilitation and inhibition increased progressively up to an age of 60 days postnatally when adult values were attained

3) During tetanisation of the ipsilateral sural nerve in newborn kittens the extensor and flexor monosynaptic potentials were usually unaffected but at later developmental stages a facilitation which attained adult values at an age of 60 days was observed

4) Extensor and flexor test potentials were usually unaffected by mechanical skin stimuli in newborn kittens In somewhat older kittens small inconstant effects were observed and in 30 days old kittens the effects were adult like

5) By recording the EMG it was found that in the newborn kittens excitatory effects on both flexor and extensor muscles could be elicited from large cutaneous areas whereas the flexors could not be inhibited at all and the extensors only from the distal planta pedis

6) The excitatory effects became gradually concealed as postnatal development proceeded and eventually reversed into inhibitory action elicitable from progressively larger skin areas The adult pattern of the excitatory and inhibitory skin areas is already present in the 2—3 weeks old kittens. The developmental changes during the first 2—3 weeks furnish an explanation for the existence of concealed reflexes in the adult stage

7) Spinalization did not change the reflex pattern until an age of 2—3 weeks

8) The general responsiveness of the muscles to skin stimuli was high in newborn kittens and decreased with increasing age In an individual newborn kitten proximal muscles were less responsive to skin stimuli than distal muscles This difference diminished with increasing age

9) The source of the most effective excitation of the motoneurones shifted postnatally from cutaneous exteroceptors to muscle proprioceptors and the postnatal changes in the cutaneous reflex effects ran in parallel with the appearance of tonic discharges from and gamma innervation of the muscle spindles When a muscle contracts in the immature kitten it will be autogenously inhibited since the muscle spindles lacking gamma activation will

cease firing The cutaneous afferent discharge on the other hand is unaffected by such mechanisms and will be effective in activating the muscles It is concluded that the postnatal change in the influence of cutaneous afferents both with regard to muscle responsiveness and amplitude alterations of monosynaptic potentials might be explained by a change brought about in the excitability of the interneurones by the increasing influence from muscle sense organs and a gradual appearance of a subliminal fringe in the moto neurone pool although a change in the ratio of excitatory and inhibitory inflows from cutaneous afferents in favour of the inhibitory inflow cannot be excluded

CHAPTER IX

GENERAL DISCUSSION

The aim of the present work was to study the mechanisms which make skin stimuli more effective than activation of muscle receptors in producing motor responses in young kittens Two main possibilities were considered 1) whether this is due to a more advanced functional maturation of the cutaneous sense organs as compared with the muscle receptors or
2) whether it is a consequence of immaturity of supraspinal regulation and segmental postural reflexes?

It was shown by morphological fibre analysis that the largest cutaneous myelinated nerve fibres are very small at birth but they gradually increase in diameter subsequently At an age of 3 months their increase in diameter is still only about 40—50 % of the total increase between birth and the adult stage

Comparisons between cutaneous nerves supplying different regions of the hindlimb of young kittens indicated that the cutaneous nerves supplying proximal areas are relatively more developed than those supplying distal structures The skin area of the distal planta pedis — the most effective skin area of the hindlimb for evoking motor responses in newborn kittens — is thus not supplied by nerve fibres that are more highly developed morphologically than those supplying other cutaneous structures of the limb The postnatal development of nerve fibres supplying the distal planta pedis is quite similar to those supplying the dorsal aspect of the foot a region that is less effective than the distal planta pedis in evoking motor responses The diameters of cutaneous nerve fibres increase more slowly than those of muscle nerves (*Skoglund* and *Romero* 1965) It was found that there is a continuous addition of myelinated nerve fibres in n suralis during about the first 2—3 months postnatally Thus there are no morphological findings indicating that the cutaneous afferents are more developed than the muscle afferents Articular afferents were found to develop at the same speed as the cutaneous afferents

A relationship between conduction velocity and nerve fibre diameter was established enabling the fibre diameter to be calculated from the conduction

velocity and vice versa A factor of 4 7 (conduction velocity/fibre diameter) was used since the well known value of 6 (*Hursh* 1939 a) was shown to be too high, especially for slow conduction velocities The conduction velocity was found to increase by 0 6 m/sec per day postnatally This value is less than that of muscle afferents i e 1 m/sec per day (*Skoglund* 1960 b) which is in accordance with the morphological findings that cutaneous afferents develop more slowly than muscle afferents

By recording from peripheral cutaneous afferent units it was found that hair units and touch units could only be brought to fire at low frequencies in response to adequate stimulation in newborn kittens and that the ability to respond with higher frequencies increased during a period of about 40—50 days postnatally Furthermore the duration of the discharge of touch units in response to a sustained stimulus was very short in newborn kittens but increased rather abruptly to adult values at the stage when the conduction velocity in the cutaneous nerves reached 20 m/sec This relationship between change of discharge duration and conduction velocity was also found in articular afferent units *Skoglund* (1960 c) also found a similar relationship in muscle sense organs It therefore seems probable that it is a phenomenon of general occurrence in the postnatal development of peripheral afferent units and that the functional change is associated with changes in the nerve fibre properties rather than in receptor structure, since the same relationship between discharge and nerve fibre properties has been found in three different structures viz muscle joint and skin Muscle afferents attain a conduction velocity of 20 m/sec earlier during postnatal development than cutaneous afferents (see above) which is the reason that the change from phasic to tonic discharges must occur earlier in muscle sense organs than in cutaneous sense organs It is therefore evident that a more advanced functional maturation of myelinated cutaneous peripheral afferent units as compared with those of muscle sense organs could not be the explanation for the effectiveness of cutaneous stimuli in producing motor responses On this basis the spinal reflex organization of the newborn kitten becomes of interest

The postnatal changes in cutaneous reflex effects had already begun before the cutaneous sense organs had reached adult functional maturation and earlier than supraspinal influences could be shown to exert their action on the spinal reflex as evidenced by the comparisons of the findings obtained before and after acute spinalization The appearances of tonic discharges from and gamma innervation of the muscle proprioceptors, on the other hand occur at the same time as the changes in the cutaneous reflex effects When a muscle contracts in the immature kitten it will be

autogenously inhibited, since the muscle spindles lacking gamma activation will cease firing but the cutaneous afferent discharge which is unaffected by such mechanisms will be effective in activating the muscles Thus the source of the most effective excitation of the motoneurones is shifted postnatally from cutaneous exteroceptors to muscle proprioceptors Furthermore the postnatal change in the effect of cutaneous afferents both with regard to muscle responsiveness and amplitude alterations of monosynaptic potentials could be explained by the gradual appearance of a subliminal motoneurone fringe A change in the ratio of excitatory and inhibitory inflows from myeli nated as well as unmyelinated afferents in favour of the inhibitory inflows also cannot be excluded though it appears unlikely Postnatally the reflex effects evoked from the skin show a progressive decrease of excitatory action with an increase of inhibitory activity In this process the dominant excitatory influences from the skin in the early developmental stage become concealed with increasing age and thus postnatal development provides an explanation for the phenomenon of concealed reflexes in adult cats

It might be expected that in general newborn kittens would have a rather poor capacity to adapt themselves to the environment due to deficient function in several systems e g the visual (the eyes are closed) and the proprioceptive systems However, it is evident that the deficient proprio ceptive mechanisms in the muscles are partly compensated for by the effectiveness of the cutaneous sense organs in evoking motor performances such as avoiding reactions and locomotion of the crawling type by means of alternating movements Thus for the period of time during development before the muscles acquire gamma innervation the firing of the motoneurones is much influenced by afferent inflow from sources other than muscle recep tors This situation is in some ways similar to that existing for those muscle groups which lack muscle spindles in the adult stage e g the afferent inflow to hypoglossal motoneurones comes from submucosal tongue sense organs connected to the lingual or glossopharyngeal nerve in cats (*Blom* 1960) and a similar situation has been reported in laryngeal muscles (*Mår tensson* 1964)

However the lack of inhibitory effects may lead to exessive motor activity in response to a persistent skin stimulus This might be counteracted in part by short lasting discharges of the immature cutaneous sense organs Remote as well as contact thermal stimuli may play a role in the activity of newborn kittens e g a decrease of the temperature gives rise to activity in the afferent cutaneous nerves and if the stimulus is sufficient it may evoke motor activity Such an ability of the afferent units might be utilized for example for locomotion and orientation in relation to the

mother cat which emits radiant heat The paucity of inhibitory mechanisms seems to be good neuronal economy in an immature organism which has to react to changes in the environment in spite of poorly developed metabolical abilities i e in the sense that in spite of the highly limited afferent impulse production of immature cutaneous sense organs(in response to a skin stimulus) the stimulus gives rise to considerable motor activity

Cutaneous reflexes are a valuable tool in clinical diagnostic work e g the Babinsky sign The mechanisms involved in cutaneous reflexes in man have been studied with neurophysiological methods (*Hagbarth* and *Kugelberg* 1958 *Kugelberg* and *Hagbarth* 1958 *Eklund Grimby* and *Kugelberg* 1959, *Hagbarth* 1960 *Kugelberg Eklund* and *Grimby* 1960 *Hagbarth* and *Finer* 1963 *Grimby* 1963) Cutaneous reflex mechanisms in babies however, have been studied relatively less with such methods The H reflex on the other hand has been studied by *Blom Hagbarth* and *Skoglund* (1964) and appears to be functionally more mature than in newborn kittens with regard to posttetanic potentiation With regard to the nerve fibre sizes of newborn babies (*Nystrom* and *Skoglund* 1965) those of the cutaneous nerves appear to correspond roughly to the developmental stage at which the discharge duration of cutaneous touch units attains adult values in kittens Though great caution must be exercised in applying findings in cats to the situation in man the present experimental findings may be useful with respect to the immature sensori motor arc in newborn infants

GENERAL SUMMARY

The work reported here is an investigation of the postnatal functional changes in the cutaneous and articular sense organs, the growth in diameter of their connecting nerve fibres and the postnatal changes in the cutaneous reflex patterns in kittens.

In the first chapter the main theme of the study is presented. It is known that in contrast to the small effects resulting from activation of muscle receptors, skin stimuli are very potent in evoking motor responses in young kittens. Two possible explanations for this phenomenon are that it is due to 1) more advanced functional maturation of the cutaneous sense organs as compared with muscle receptors or 2) a lack of supraspinal regulation and absence of segmental postural reflexes.

The anatomy of the cutaneous nerves in the hindlimb and the nomenclature used are presented in Chapter II. In Chapter III the segmental origin of different hindlimb cutaneous nerves was studied by electrical stimulation and recording. The composition of the segmental origin varies considerably between animals and the constituent fibres of a nerve do not always pass through consecutive rootlets. It was found, however, that some fibres of a particular nerve often entered a certain rootlet. The latter finding is useful when single dorsal root fibres contributing to a certain nerve must be identified. Some observations on the distribution of hindlimb dermatomes are reported in Chapter IV. In contrast to an earlier investigation it was found that the S1 dermatome usually reaches distally down to the middle of the foot.

In Chapter V the postnatal development of the fibre compositions of cutaneous and articular nerves were studied in osmium stained sectioned material. The size of the myelinated nerve fibres of cutaneous nerves was very small in the youngest kittens. The diameter range of the nerve fibres increased during postnatal development and the increase of the largest fibres at an age of 90 days was only about 40—50 % of the total increase between birth and the adult stage. The development of the articular nerve fibres (knee joint) was similar to that of the cutaneous nerves of the hindlimb. The diameter increase of the largest cutaneous and articular nerve fibres was

slower than that of muscle nerves Comparisons between different cutaneous nerves gave results consistent with a cephalocaudal and proximo-distal development

Chapter VI deals with postnatal changes of the conduction velocity in the sural nerve The conduction velocity increased by approximately 0 6 m/sec per day or 1 mm/sec per one mm increase of the femur length The conduction time for a stretch of nerve equal to the femur length decreased postnatally The developmental stage at which a nerve attains a maximal conduction velocity of 20 m/sec is of particular interest with regard to the cotemporaneous changes in the physiological properties of the sensory units The largest fibres of such a nerve were calculated to have a diameter of 4 3 μ A factor of 4 7 was used for this calculation because Hursh s factor of 6 was shown to be too high especially for slow conduction velocities

In Chapter VII the postnatal changes in the properties of the discharge from cutaneous and articular peripheral afferent units were investigated By means of single unit recordings in the dorsal root it was found that hair units and touch units could only be brought to fire at low frequencies in response to mechanical stimulation of the skin in newborn kittens and that the ability to respond with higher frequencies increased progressively up to an age of about six weeks The duration of the discharge of touch units in response to a sustained constant mechanical stimulus is very short in newborn kittens and the duration abruptly increases to adult values at the stage when the conduction velocity of the cutaneous nerve reaches 20 m/sec The same relationship between change of discharge duration and fibre size applies for articular afferent units and since a similar relation has been described in muscle sense organs it appears to be a general phenomenon in the development of peripheral afferent units Electrical stimulation of the skin in the receptive fields of hair and touch units indicated that the ability of the immature nerve fibres to fire with a high frequency is lower than that of adult fibres In contrast to the property of touch units in adult cats immature units gave discharges of shorter duration and with a smaller total number of spikes in response to strong stimulation than in response to weak stimulation The articular units behaved similarly in this respect

In Chapter VIII the postnatal changes in the cutaneous reflexes were analyzed By recording monosynaptic test potentials and conditioning stimulation of 1) a cutaneous nerve or 2) the cutaneous sense organs (mechanically) it was found that a manifest facilitation was not obtained until an age of 5—6 days postnatally Subsequently facilitation and inhibition gradually increased in magnitude and in 60 days old kittens the findings were adult like

By means of EMG recordings it was found that in the newborn kitten excitatory effects in both flexor and extensor muscles could be elicited from large cutaneous areas No skin area was found which inhibited the flexor activity induced from an excitatory area Extensor activity could only be inhibited from the distal planta pedis The excitatory effects from a certain skin area gradually became concealed during postnatal development and reversed into inhibitory action elicitable from progressively larger skin areas The adult pattern of the excitatory and inhibitory skin areas is present in 2—3 weeks old kittens The developmental changes thus give an explanation for the concealed reflex present in the adult stage Spinalization did not change the reflex pattern until an age of 2—3 weeks postnatally indicating that there is a postnatal change in supraspinal influences on the spinal reflexes that may constitute one factor responsible for the excitability changes at the spinal level The source of the most effective excitation of the motoneurones is shifted postnatally from cutaneous exteroceptors to muscle proprioceptors and the postnatal changes in the cutaneous reflex effects run in parallel with the appearance of tonic discharges from and gamma innervation of the muscle spindles When a muscle contracts in the immature kitten it will be autogenously inhibited since the muscle spindles lacking gamma activation will cease firing The cutaneous afferent discharge on the other hand is unaffected by such mechanisms and will be effective in activating the muscles It is concluded that the postnatal change in the influence of cutaneous afferents both with regard to muscle responsiveness and amplitude alterations of monosynaptic potentials may be explained by a gradual change of the excitability of the interneurones and a gradual appearance of a subliminal fringe in the motoneurone pool although a change in the ratio of excitatory and inhibitory inflows from cutaneous afferents in favour of the inhibitory inflow cannot be excluded

Chapter IX is a general discussion of some of the results reported in the preceding chapters As regards the main questions posed it can be concluded that a greater efficiency in the discharge of the cutaneous sense organs as compared with muscle sense organs is not the explanation for the effectiveness of cutaneous stimuli in producing motor responses in young kittens which must therefore be due to the functional organization of the spinal cord It is probable that the postnatal changes in the cutaneous reflex patterns also can be attributed to functional changes in the spinal cord although a change in the ratio between excitatory and inhibitory inflows from cutaneous afferents cannot be excluded

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ACTA PHYSIOLOGICA SCANDINAVICA

STUDIES ON THE METABOLISM OF 1,4-^{14}C-SPERMIDINE AND 1,4-^{14}C-SPERMINE IN THE RAT

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PREFACE

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MARTTI SIIMES

CONTENTS

INTRODUCTION

The polyamines spermidine ($H_2N(CH_2)_3NH(CH_2)_4NH_2$) and spermine ($H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$) are aliphatic nitrogenous bases widely distributed in various organisms. The existence of polyamines has been known for a long time probably since the 17th century (LEWENHOECK 1678). Only during the last decade has an increasing amount of knowledge been accumulated about these compounds. In this context some light will be thrown on the occurrence possible functions biosynthesis and degradation of polyamines.

Occurrence of polyamines. Even as early as 1925 DUDLEY and ROSENHEIM determined the spermine of several animal tissues. Human semen ox liver kidneys and spleen were reported to contain relatively large amounts of spermine. ROSENTHAL and TABOR (1956) analysed both the spermidine and spermine contents of various organs in a number of laboratory animals. A detailed study of the concentrations of polyamines in different rat tissues was reported by JÄNNE RAINA and SIIMES (1964). In three month old rat the concentration of spermidine per gram of tissue wet weight varied in different tissues from 100 to 1 600 mμmoles and that of spermine from 160 to 900 mμmoles. The authors also observed in every studied tissue a decrease in the spermidine concentration in relation to age e g from 2 000 to 600 mμmoles per gram of liver from birth to nine months. The fall was most marked during the first month of life. In some tissues there was a rise in the spermine concentration during the first month but in general the changes in this amine with age were small. Recently the brain polyamine contents of various mammals have also been analysed (JÄNNE RAINA and SIIMES 1964 SHIMIZU KAKIMOTO and SANO 1964 PERRY HANSEN FOULKS and LING 1965 MICHAELSON 1966). A decrease in the spermidine concentration of mouse brain during the perinatal period was established by SHIMIZU KAKIMOTO and SANO (1965a). Only small amounts of polyamines about five mμmoles per millilitre have been observed in the blood cells as pointed out by RAINA (1962a) and SHIMIZU KAKIMOTO

and SANO (1965b) and none in human blood plasma (ROSENTHAL and TABOR 1956 RAINA 1962a BACHRACH and ROBINSON 1965)

Relatively high concentrations of polyamines and especially putrescine a spermidine precursor have been observed in various microorganisms E coli has been reported to contain 15 µmoles of putrescine and 1 5 µmoles of spermidine per gram (TABOR ROSENTHAL and TABOR 1958) However the polyamine concentrations in different bacteria vary with the pH and other cultural conditions (TABOR ROSENTHAL and TABOR 1958, DUBIN and ROSENTHAL 1960a RAINA and COHEN 1966) A great variety of polyamine is found in different strains Part of the putrescine and spermidine are in the acetylated forms (DUBIN and ROSENTHAL 1960a, RAINA and COHEN 1966 COHEN and RAINA 1967) It is noteworthy that the T even type bacteriophages of E coli contain very high concentrations of putrescine and spermidine per gram (AMES and DUBIN 1960) In addition various plants are known to contain polyamines as discussed by TABOR and TABOR (1964b)

Possible functions of polyamines Several authors have made efforts to find some function for polyamines in cells In 1949 HERBST and SNELL were the first to find that spermine is a growth factor for *Hemophilus parainfluenzae* Even now the mechanism by which spermine influences growth is not definitely established On the other hand spermine is also known to inhibit both the growth (RAZIN and ROZANSKY 1959) and the protein synthesis of *Staphylococcus aureus* (FRIEDMAN and BACHRACH 1966) and of Walker carcinosarcoma cells (GOLDSTEIN 1965) The studies *in vitro* have shown that spermine spermidine and putrescine stimulate the incorporation of amino acid into protein (HERSHKO AMOZ and MAGER 1961 MARTIN and AMES 1962) possibly as a result of the stabilization of 70S ribosomes by polyamines (ZILLIG KRONE and ALBERS 1959 COHEN and LICHTENSTEIN 1960 COLBOURN WITHERSPOON and HERBST 1961 MARTIN and AMES 1962) in the presence of suboptimal magnesium ion concentration (HERSHKO AMOZ and MAGER 1961) It has also been demonstrated that at low concentrations in the medium spermine stimulates and at high concentration inhibits polypeptide synthesis by a subcellular system derived from L 1210 mouse ascites leukemia (OCHOA and WEINSTEIN 1964) The inhibitory effect of spermine is dependent upon the concentrations of both sRNA and mRNA but not of microsomes (OCHOA and WEINSTEIN 1965)

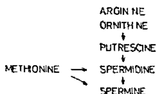
Many close associations have been reported between polyamines and nucleic acids Polyamines precipitate nucleic acids *in vitro* (TABOR 1962) They have the ability of protecting helical DNA against thermal denaturation (MANDEL 1962) In the presence of polyamines an increased resistance to thermal denaturation of RNA has also been noticed (GOLDSTEIN 1966) The polyamines have a strong affinity with the cellular constituents of acidic groups A number of reports indicate that polyamines can stabilize and protect ribosomes mitochondria cell membranes bacteriophages and nucleic acids themselves The association between polyamines and cellular structures and nucleic acids has been discussed in detail in a review by TABOR and TABOR (1964b) and very recently by COHEN and RAINA (1967) The latest studies cited below have thrown considerable light on the role of polyamines in relation to nucleic acids RAINA and COHEN (1966) observed that the ratio of total polyamine nitrogen to RNA phosphate varied within fairly narrow limits (0 21—0 23) in growing cells of *E coli* Some studies have shown that spermine spermidine and putrescine *in vitro* somewhat stimulate the initial rate of RNA synthesis and allow also the action of RNA polymerase to continue (KRAKOW 1963 FOX ROBINSON HASELKORN and WEISS 1964 FOX and WEISS 1964) The DNA primed RNA polymerase reaction was progressively stimulated by graded concentrations of spermine to 10^{-3}M (O BRIEN OLENICK and HAHN 1966) The effect *in vivo* might be similar In fact this kind of effect has been demonstrated by RAINA and COHEN (1966) The exogenous putrescine 150 mM caused a slight increase and the exogenous spermidine 20 mM or more a marked increase in the uracil incorporation into RNA These direct observations on the effects of polyamines were made in the polyauxothrophic strain of *E coli* during an amino acid starvation A rise in the RNA synthesis has also been obtained by spermine in *E coli* (MILLS and DUBIN 1966) and in a tissue culture (GOLDSTEIN 1965) Recently SIMON, COHEN and RAINA (1966) studied the inhibition of RNA synthesis in *E coli* and they noticed that spermidine reversed the inhibition of RNA synthesis caused by Jevorphanol

Spermine (10^{-4}M) has been demonstrated as an inhibitor of the DNA polymerase activity *in vitro* (O BRIEN OLENICK and HAHN 1966) The latest observations by BREWER and RUSCH (1966) indicate the stimulatory effect of spermine when administered together with DNA on the DNA polymerase reaction in the isolated nuclei of a plasmodia This effect was most noticeable in the nuclei isolated 1 5 hours after mitoses

Only a slight stimulation was obtained with spermine and DNA used separately under the same experimental conditions

B i o s y n t h e s i s o f p o l y a m i n e s The first experiments con cerning the biosynthetic pathways of polyamines in microorganism wer reported in 1956–1958 Labelled putrescine and ornithine were shown to serve as precursors of the 4 carbon chain (TABOR ROSENTHAL and TABOR 1956 1958) and methionine of the 3 carbon moieties in sper midine and spermine (GREENE 1957 TABOR ROSENTHAL and TABOR 1957 1958) In this reaction methionine is used in the form of S adenosyl methionine (CANTONI and DURELI 1957) which is decarboxylated for the spermidine synthesis (TABOR ROSENTHAL and TABOR 1956 1957 TABOR and TABOR 1960 TABOR 1962a b) MORRIS and PARDEE (1965 1966) have shown that biosynthesis of putrescine occurs directly through the decarboxylation of ornith ne or through the formation of agmatine from arginine in E c o l i Purification of the enzymes cata lysing the steps in the spermidine biosynthesis from putrescine and met hionine has also been reported by TABOR and TABOR (1960) and TABOR (1962 a b) On the other hand spermidine could not substitute for put rescine as an acceptor of propylamine moiety (TABOR 1962b) Accordingly nothing is known about the enzymatic mechanisms in the biosynthes s of spermine from spermidine

Polyamine biosynthesis has also been demonstrated by ^{14}C labelled precursors in chick embryo by RAINA (1962b 1963) and recently in rat tissues by RAINA (1964) JÄNNE and RAINA (1966) and RÄIHÄ JÄNNE and SUIHKONEN (1967) The same precursors were described in rat ti sues as in microorganisms illustrated as follows



The synthesis of polyamines has also been studied in rat liver regenerat ing after partial hepatectomy The incorporation of radioactivity from labelled arginine (JÄNNE and RAINA 1966) and especially from methio nine (RAINA JÄNNE and SIIMES 1966) into spermidine has been shown to increase greatly in the liver after partial hepatectomy DYKSTRA and HERBST (1965) reported that the conversion of ^{3}H putrescine into sper

midine in the regenerating rat liver was almost double as early as two hours after operation However JÄNNE and RAINA (1966) po nted out that there was a fall in the specific activity of spermidine after administration of labelled putrescine RAINA JÄNNE and SIIMES (1965 1966) and DYKSTRA and HERBST (1965) observed an increase in the spermidine concentration of the liver gram most noticeable 64 to 72 hours after partial hepatectomy A decrease in the spermine concentration per gram of liver at 32 hours was also demonstrated The ratio of total spermidine-nitrogen to ribonucleic acid phosphate appeared to be quite constant during liver regeneration There was also a correlation between the concentrations of deoxyribonucleic acid and spermine throughout the period of regeneration (RAINA JÄNNE and SIIMES 1965 1966) The miscellaneous changes in the metabolism of regenerating liver have been reviewed by BUCHER (1963)

Ethionine is a toxic methionine analogue which changes the metabolism of tissues in many respects It has been reported to inhibit the synthesis of RNA (VILLA TREVINO SHULL and FARBER 1963b FARBER SHULL VILLA TREVINO LOMBARDI and THOMAS 1964) and in addition stimulation effects have also been shown (TURNER and REID 1964) In its role ethionine is known to inhibit protein synthesis lower the activities of several enzyme systems and cause hepatomas etc The interesting material concerning the effects of ethionine on tissues has been reviewed by STEKOL (1963)

Ethionine being a methionine antagonist has been used to obtain information on the metabolism of polyamines RAINA (1963) reported that ethionine treatment reduced both the spermine concentration and the incorporation of labelled methionine into this polyamine in the developing chick embryo During daily ethionine administrations the concentration of spermidine rises in rat liver (RAINA JÄNNE and SIIMES 1964 KREMZNER and STARR 1966) and that of spermine decreases (RAINA JÄNNE and SIIMES 1964) These effects have been attributed to the formation of S adenosylethionine (MODY BULBA HOLOWECKY and STEKOL 1963 RAINA JÄNNE and SIIMES 1964 SHULL McCONOMY VOGT CASTILLO and FARBER 1966) with a fall in the adenosyl nucleotides (STEKOL MODY BEDRAK KELLER and PERRY 1960 CALDARERA BUDINI BARBIROLI and RABBI 1962 SHULL 1962 VILLA TREVINO SHULL and FARBER 1963a BARTELS and HOHORST 1963 MODY BULBA HOLOWECKY and STEKOL 1963 RAINA JÄNNE and SIIMES 1964) The effect of ethionine on the concentrations of polyamines

is dependent upon the daily dosage the cessation of treatment and the tissue which has been analysed (SIIMES RAINA and JÄNNE und r preparation)

Degradation of polyamines HIRSCH (1953a b) was the first to discover in the blood plasma of the sheep and the ox an enzyme which caused rapid oxidative deamination of spermine and which also acted on spermidine He called this enzyme spermine oxidase Using enzyme preparations it was reported that spermine is first converted into spermidine which is then further oxidized (TABOR and ROSENTHAL 1956b BACHRACH and BAR OR 1960 YAMADA and YASUNOBY 1962 UNEMOTO 1963) The formation of ammonia hydrogen peroxide and aldehydes during oxidation was also demonstrated (ROULET and ZELLER 1945 HIRSCH 1953 a b TABOR and ROSENTHAL 1954) Recently TABOR TABOR and BACHRACH (1964) have shown using purified enzyme preparations that polyamine aldehydes are the only products of this oxidation This discovery does not agree however with those mentioned above i e that free spermidine is formed from spermine by plasma oxidase

As early as 1944 SILVERMAN and EVANS noticed that polyamines were rapidly oxidized by Pseudomonas aeruginosa Some reports show that variou bacteria are able to degrade spermine to spermidine (RAZIN BACHRACH and GERY 1958 RAZIN GERY and BACHRACH 1959 BACHRACH PERSKY and RAZIN 1960) A recent report indicates that spermidine is further degraded to putrescine and 3 aminopropionalaldehyde in Pseudomonas aeruginosa (PADMAHABHAN and KIM 1965) BACHRACH (1962 a b) purified an enzyme from Serratia marcescens which oxidized spermidine to 1 3 diaminopropane and Δ1 pyrroline Thi enzyme did not act on spermine and it was reported to be different from the plasma oxidase

The nonoxidative metabolism of polyamines in bacteria has also been studied Acetylation of polyamines has been pointed out by DUBIN and ROSENTHAL (1960) ROSENTHAL and DUBIN (1962) and RAINA and COHEN (1966) The products are in E coli monoacetylputres ine two isomeric forms of monoacetylspermidine derived from spermidine and mono and diacetylspermine from spermine (DUBIN and ROSENTHAL 1960) The glutathione conjugates of the polyamines have also been reported (DUBIN 1959 DUBIN and ROSENTHAL 1960 b TABOR TABOR and de MEIS 1966)

Little information is available on the degradation of polyamines in

animal tissues The only evidence for the degradation of spermine to spermidine in animals has been observed by ROSENTHAL and TABOR (1956) when they studied the distribution and excretion of polyamines After the administration of nonlabelled spermine to rats mice and rabbits 4 to 8 per cent of the dose was excreted in the urine as spermidine during the first day When spermidine was injected no spermine appeared in the urine The concentration of spermidine was also found to increase slightly in a few tissues after administration of spermine

More detailed knowledge of the metabolic role of polyamines and their metabolism has been reviewed by TABOR TABOR and ROSENTHAL (1961) BLASCHKO (1962) RAINA (1963) TABOR and TABOR (1964) BEER ROSIEK and SABLINSKY (1966) and COHEN and RAINA (1967)

The purpose of the present investigation was to study the metabolic fate of exogenously administered spermidine and spermine in rat tissues by 1 4 ^{14}C labelled polyamines *) The study of polyamine interconversion in rat tissues *in vivo* formed the main part of this work Evidence on the transformation of polyamines has been reported in bacteria but in animal tissues attention has not been paid to this section of polyamine metabolism

The polyamine concentrations in the rat tissues are dependent upon the tissue and the age of the rat Relatively high spermidine concentrations have been noticed in every young rat tissue studied The concentration of spermidine also varies considerably in different tissues obtained from rats of the same age For instance the concentration of spermidine and the molar ratio spermidine/spermine is fairly low in the kidneys and unusually high in the pancreas This data prompted a study of polyamine interconversion in relation to age in the pancreas and kidneys as well as in the liver and intestine

An earlier study of the biosynthesis of polyamines has also been made in regenerating rat liver after partial hepatectomy During the development of regeneration the biosynthesis and the concentration of spermidine is known to be increased In addition the treatment with ethionine causes characteristic changes in the polyamine concentrations These variables also formed a basis for the study of the interconversion of administered polyamines

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*) In literature not only spermidine and spermine are regarded as polyamines but often also diamines such as putrescine and cadaverine However in this text only spermidine and spermine will be referred as polyamines

In this study the radioactive amines were given exogenously to the rats To obtain a picture which would be as close as possible to the metabolism of the endogenous polyamines only trace amounts of these compounds with high specific activity was used This handling did not essentially change the pool of endogenous polyamines On the other hand there is only poor evidence on whether the metabolism of exogenous poly amines besides those of synthesized forms an essential element in the total metabolism of polyamines e g by absorbing from the intestine

Knowledge on the turnover of exogenously administered polyamines is also lacking Accordingly it was considered necessary to study also the distribution and elimination of exogenously injected polyamines in the tissues and their possible routes of elimination in rat organisms as for example through the lungs into the urine and into the intestine It was hoped that a better understanding of the fate of injected 1 4 ^{14}C labelled polyamines would afford some more information on the metabolism and the metabolic role of these amines

MATERIALS

Animals The rat was chosen as the experimental object for the present study The biosynthetic pathways of polyamines in rat tissues have recently been studied in this laboratory This animal was thus considered to be suitable for use in the study of the fate of these amines The experiments were performed *in vivo* with female Wistar strain rats The age of the animals varied from the newborn to 30 weeks The newborn rats were less than 6 hours old at the commencement of the experiment The animals were fed unless otherwise indicated *ad libitum* with a standard diet manufactured by Hankkija Oy Helsinki Finland Variations in the weight of rats in different groups was kept small and it never exceeded ±10 per cent of the mean value Each group comprised 2 to 8 rats and normally 4 in the isotope experiments

Reference standards Spermidine phosphate spermine tetrahydrochloride and putrescine dihydrochloride were commercial analytical grade preparations from Mann Research Laboratories Inc New York U S A

Reagents The reagents and solvents used in these experiments were of analytical grade They were mainly manufactured by E Merck Darmstadt Germany

Isotopes Two ^{14}C-labelled compounds now commercially available were used Spermidine 1,4 ^{14}C tetrahydrochloride [(aminopropyl) tetramethylene-1 4 ^{14}C diamine] 4HCl and spermine 1 4 ^{14}C tetrahydrochloride [(bis (aminopropyl) tetramethylene 1 4 ^{14}C-diamine] 4HCl were both purchased from the New England Nuclear Corporation Boston Massachusetts U S A The specific activity of spermidine was 6 6 mC/mmole and that of spermine 4 2 mC/mmole The stock isotope was stored at a temperature of −18°C

METHODS

Handling of the animals

Labelled polyamines were usually injected intraperitoneally to the rats Subcutaneous and oral administration were also used in a few experiments The injections were made in a volume of 0 2 to 1 0 ml in 0 9 per cent sodium

chloride The rats were killed by decapitation The tissues were weighed in cool and homogenized in 0 1 N hydrochloric acid The details of the method of preparation and handling of the tissues have been described previously by RAINA (1963)

Collection of the urine

The urine was collected from the rats fed ad libitum on a porcelain plate under the cage Before collection about 10 ml of 0 1 N hydrochloric acid was placed on the plate which was covered by a metal web to prevent a contamination by feces The urine which has been diluted with hydrochloric acid was filtered and evaporated to a volume of 8 ml at room temperature These samples were treated in a similar way as the tissue homogenates (RAINA 1963)

Collection of carbon dioxide

Respiratory CO_2 was collected and its radioactivity measured after the injection of labelled polyamines to rats A rat fed ad libitum was placed immediately after injection in a glass jar into which incoming air came through four washing tubes each containing 50 ml of 1 N sodium hydroxide The exhaled CO_2 was collected in five imilar tubes by water suction After collection which lasted for 1 to 3 hours the sodium bicarbonate in these five tubes was combined and mixed An aliquot of 5 ml was placed in an Erlenmeyer flask with a centre well In addition 1 ml of hyamine (Packard Instrument Company Inc Illinois U S A) was placed in the centre wells The flasks were tightly closed with rubber stoppers 2 ml of 4 N hydrochloric acid was injected through the stoppers into the sodium bicarbonate solution to liberate CO_2 Twelve flasks at a time were slowly haken in a mechanical shaker for one hour at room temperature The hyamine was transferred into 9 ml of toluene scintillation liquid with which the centre wells were carefully rinsed several times The controls without radioactivity were treated in a similar way Freshly prepared sodium hydroxide and hyamine were used

Partial hepatectomy

Partial hepatectomy of the rats was performed according to HIGGINS and ANDERSON (1931) The rats were anaesthesized with aether A single thread ligature was tied few millimetres distally from the pedicles and the lobes removed These three liver lobes together amounted to about two-thirds of the liver weight The operation appeared to have no fatal results if properly performed The abdominal cavity was closed tightly in two layers After decapitation when the residual livers were analysed only the two corresponding lobes were analysed from both the operated and control rats

Quantitative determination of polyamines and putrescine

Tissue polyamines were separated by paper electrophoresis of an alkaline butanol extract from the deproteinized ample Amido black was used as the colour reagent This sensitive method has been described by RAINA (1963) Putrescine was analysed quantitatively from the same tissue samples according to RAINA and COHEN (1960) using cadmium ninhydrin as the colour reagent

Radioactivity measurements

The radioactivity in putrescine spermidine and spermine can be separated by electrophoresis on paper (RAINA 1963) The separation of polyamines for radioactivity measurements was obtained with 0 1 M citric acid buffer pH 3 6 in 3 hours and that of putrescine at pH 4 3 in 2 hours The fractions were visualized by spraying with 0 1 per cent ninhydrin reagent From the paper strips the polyamine fractions and 2 blank fractions were cut out one blank far from the cathode side of the paper and one between the two polyamine fractions The pieces of papers containing the radioactivities of the polyamines and blank fractions were placed directly in the scintillation flasks A day after the addition of 2 ml of scintillation liquid (3 g 2 5 diphenyloxazole and 0 1 g 1,4 bis -(4 methyl phenyloxazolyl) benzene/1 000 ml toluene) obtained f om Packard Instrument Company Inc Illinois U S A the radioactivity was measured in a Packard Tri Carb Liquid Scintillation Spectrometer Model III

In this study 1 0 μC of 1 4 ^{14}C spermidine standard on paper gave around 1 040 000 counts per min and 1 4 ^{14}C spermine about 1 080 000 counts per min These standards were measured in a similar way and counted during every series of experiments

All radioactivity measurements in this work were performed on the paper by the above mentioned technique except for that of carbon dioxide The hyamine bound $^{14}CO_2$ was diluted with toluene scintillation liquid too but the counting efficiency was about 15 per cent higher in these measurements than in the measurements directly from the paper

Control of the radioactivity measurements

The paper electrophoretic technique produced good separation between spermidine and spermine radioa tivities During the optimal conditions of the routine method 1 4 ^{14}C spermidine diluted in 20 mμmoles of spermidine and spermine produced a contamination of about 0 5 per cent in the fraction between polyamines and about 0 2 per cent in the spermine fraction relative to the radioactivity on spermidine The contamination from the labelled spermine in the spermidine fraction was only 0 05 to 0 01 per cent of the radioactivity in the spermine fraction These contaminations were considered to be without practical significance in the present work

The Rf values of 1 4 ^{14}C spermine and spermidine which were formed in the liver with some solvent systems as given in Table I were equal to those of the endogenous polyamine fractions

The only radioactive compounds were spermidine and spermine which were found in the liver samples one day after an intraperitoneal injection of a 1 4 ^{14}C polyamine demonstrated as follows According to the routine method described by RAINA (1963) the trichloroacetic acid supernatant of the tissue homogenate was extracted with aether 3 times The aether extracts were combined evaporated at room temperature and diluted to a small volume which was placed totally on the paper dried and measured in toluene scintillation solution Only trace of radioactivity wa found in this sample The trichloro acetic acid bound protein was diluted in sodium hydroxide and its radioactivity was measured in Bray s solution (BRAY 1960) Here again only small amounts of radioactivity was detected As the next step butanol extraction of the samples was performed from both alkaline and acid solution The recovery of the radioactivity after the alkaline butanol extraction was essentially the same as reported by RAINA (1963) Only around 5 per cent of the radioactivity was recovered from the acid butanol Alkaline butanol soluble radioactivity was found in the electrophoretic fractions of polyamines spermidine and spermine

The radiopurity of the spermidine and spermine fractions was also tested from the liver samples [illegible] days after injection of labelled spermidine or spermine as follows The polyamines were separated by a preparative ascending paper chromatography with the solvent VII see Table I On the paper narrow strips were stained with 0 1 per cent ninhydrin reagent using a micropipette Following these small guide fractions the unstained spermidine and spermine fractions were separately cut off and eluted After alkaline butanol extraction (RAINA 1963) the samples were subjected to paper electrophoresis After electrophoretic separation the specific activities of spermine and spermidine were essentially the same as after the first separation by chromatography

The radioactive spermidine derived from 1 4 ^{14}C spermine and isolated by preparative paper electrophoresis as described above was also diluted with nonlabelled spermidine tetrahydrochloride The preparation was recrystallized in ethanol water (JACKSON and ROSENTHAL 1960) The precipitate was collected by centrifugation wa hed several times with 99 9 per cent ethanol and dried *in vacuum* The specific activity of spermidine was essentially the same at the beginning of the experiment a after the crystallization

Further experiments were carried out to eliminate the possible occurrence of polyamine derivates on the electrophoretic fractions of spermidine and sper mine RAINA (personal communication) has observed that acetyl derivates of polyamines and putrescine which are normal constituents in *E coli* run in the same area as polyamines themselves in the paper electrophoresis during the routine procedure The radioactive polyamine fractions were separately hydro lysed in strong acid The samples were kept in 6 N hydrochloric acid in sealed tubes at a temperature of 100 and 120 ° C for 12 hours The specific activities of labelled polyamines which were found in the liver were essentially the same after the hydrol sis Aldehyde derivates of the polyamines were excluded

by treating the liver homogenate 1 and _ days after and the blood 70 min after the intraperitoneal injections of 1 4 ^{14}C polyamines with odium borohydride reagent overnight The details of this procedure have been described by TA BOR TABOR and BACHRACH (1964) It is likely according to above mentioned

Table I Chromatography of liver polyamines and putrescine Rf values were determined by staining the chromatograms with ninhydrin or amido black (RAINA 1963) reagents The samples were first run with paper electrophoresis or solvent VI in a thin layer The amine fractions were then eluted and rerun with other solvents

System	Rf values		
	Spermine	Spermidine	Putrescine
Sol I[1])	0 30[2])	0 60[2])	1 00[2])
Sol II[3])	0 02	0 07	
Sol III[3])	0 25	0 38	
Sol IV[3])	0 18	0.25	0 34
Sol V[3])	0 2_	0 34	0 44
Sol VI[4])	0 38	0 46	

Sol I n butanol glacial acetic acid water 50 25 _5 (FINK CLINE and FINK 1963)
Sol II n butanol methylethylketone formic acid water 40 30 15 15 (FINK CLINE and FINK 1963)
Sol III iso propanol water conc hydrochloric acid 65 18 4 16 6 (FINK CLINE and FINK 1963)
Sol IV n butanol acetic acid pyridine water 40 10 10 20 (DUBIN and ROSENTHAL 1960)
Sol V n propanol conc hydrochloric acid water 30 10 10 (DUBIN and ROSENTHAL 1960)
Sol VI ethylene glycol monomethyl ether propionic acid water - 70 15 15 (RAINA 1963)
Sol VII [3]) sol VI in saturated sodium chloride (RAINA 1963)

[1]) Descending paper chromatography Whatman No 1 paper Running time 48 hours
[2]) Correlated with Rf values of putrescine which was run about a distance of 30 cm
[3]) Ascending paper chromatography with paper of Whatman No 1 Run a distance of 18 to 20 cm
[4]) Thin layer chromatography Kieselgel G 70 g — water 100 ml The gel was 0 5 mm thick Run a distance of 15 cm

evidences that there are no acetyl or aldehyde derivates with the label in liver after injection of labelled polyamines

During the early regeneration of the liver the incorporation of 1 4 ^{14}C spermidine into putrescine was stimulated A few studies were carried out on the identification of the radioactivity of the electrophoretic fraction of putrescine The used 1 4 ^{14}C spermidine solution in 20 mμmoles of putrescine spermidine and spermine about 10^{6} cpm per electrophoretic paper strip gave practically no contamination on the putrescine fraction In the normal liver 3 hours after *an injection of 1 4 ^{14}C spermidine (5×10^{5} cpm) the radioactivity* on the putrescine fraction barely exceeded the background level Six hours after partial hepatectomy when the similar injection had been given 3 hours earlier the counts on putrescine were about 4 fold the background. From the pooled sample derived from the partially hepatectomized livers after injection of labelled spermidine the putrescine fraction was separated from the polyamines by a preparative chromatography with solvent VII see Table I The Rf values of the putrescine fraction are given with 3 solvent systems of different chromatographies in Table I No loss of counts compared to the primary paper electrophoretic fraction was found in the putrescine of the chromatographies The sodium borohydride treatment (TABOR TABOR and BACHRACH 1964) did not show any effect on the counts in the putrescine fraction either

Statistical procedures In general the presented values are arithmetic means In some instances also the range of values in a group is given The detailed statistical analyses were not considered essential in this study because the variation in the values inside a group was small enough to show the differences found Usually the deviation in the separate values was less than ± 10 per cent of the mean In some experiments demonstrating the results of ethionine treatment carbon dioxide collection radioactivities in the blood and absorption from the intestinal tract somewhat larger deviations were noticed They were mostly less than ± 20 per cent of the mean value of a group

EXPERIMENTAL PART

FATE OF 1,4-^{14}C-POLYAMINES IN TISSUES

DISTRIBUTION OF 1,4-^{14}C-POLYAMINES IN TISSUES

Some aspects of the metabolism of polyamines in the organism may be conviniently studied by following the fate of the exogenously administered labelled polyamines. First the total distribution of the injected polyamines was studied in some tissues. Comparisons were also made using different routes of administration.

The distribution of 1,4-^{14}C-spermidine in certain tissues after intraperitoneal injection is presented in Table II. 1.0 µC 1,4-^{14}C-labelled spermidine (about 10^6 cpm) was injected into one and two month old rats and they were decapitated 12 hours after injection. Table II indicates that a great part, 71.6 per cent, of the injected radioactivity was still unchanged as ^{14}C-spermidine in the whole animal 12 hours after an

Table II. *Distribution of injected 1,4-^{14}C-spermidine in tissues. Spermidine radioactivities per organ and a percentage of the injected dose have been determined 12 hours after an injection. The dose was 1.0 µC (about 10^6 cpm) per rat. The age of the rats was one month. Means and ranges of 4 animals in a group are presented.*

Tissue	Distribution of 1,4-^{14}C-spermidine	
	cpm per organ	per cent of dose
Liver	207 000 (182 000 — 231 000)	20.0
Kidneys	18 600 (16 500 — 20 000)	1.8
Intestine	286 000 (234 000 — 305 000)	27.6
Rest of tissues	230 000 (198 000 — 242 000)	22.2
Total	741 000	71.6

injection Nearly one half of the given activity was recovered merely from the liver and intestine although the total weight of these two tissues re presented about 15 per cent of the rat weight 1 8 per cent was found in the kidneys and 22 2 per cent in the rest of the rat tissues

The distribution of the given 1 4 ^{14}C spermidine was essentially similar after subcutaneous injection Both liver and intestine contained about 23 per cent each of the injected dose 12 hours after this administration The fairly similar distribution of the exogenous ^{14}C spermidine in the intestine and liver both after intraperitoneal and subcutaneous injections gives reason to believe that this compound is distributed through the blood circulation into the e intraperitoneal organs

In a comparable study with 1 4 ^{14}C spermine 1 0 µC (about 10^6 cpm) was injected to rats 12 hours after intraperitoneal injection the radio activity was analysed in the same tissues Table III shows that somewhat

Table III Distribution of injected 1 4 ^{14}C spermine in tissues A comparable study to that presented in Table II but using spermine

Tissue	Distribution of 1 4 ^{14}C-spermine	
	cpm per organ	per cent of dose
Liver	30_ 000 (285 000 — 330 00)	28 0
Kidneys	60 500 (53 800 — 64 _00)	5 6
Intestine	_68 000 (248 000 — 293 000)	24 8
Rest of tissues	233 000 (221 000 — 267 000)	21 6
To al	863 500	80 0

more 80 0 per cent of the given dose was recovered as unhanged ^{14}C spermine in total rat This finding suggests a slower turnover rate of exogenously administered 1 4 ^{14}C spermine than 1 4 ^{14}C spermidine The dis tribution of the unmetabolized ^{14}C spermine in the tissues differs from that of ^{14}C spermidine too In the liver (28 0 per cent) and especially in the kidneys (5 6 per cent) proportionally more was found than after injection of ^{14}C spermidine

Labelled polyamines were also administered *per os* 0 5 µC (5×10^5 cpm) in 0 5 ml of 0 9 per cent sodium chloride was given through a thin cathetre into the stomach of the rats The radioactivity in the intestinal

Table IV Absorption of labelled polyamines after peroral administration 0 5 µC (about 5 x 10^{8} cpm) of 1 4 ^{14}C labelled spermidine or spermine were administered per os After 1 to 16 hours the rats were sacrificed 3 weeks old rats were used Means of 2 to 3 animals in a group are shown Total radioactivities per organ are presented

Time after administration in hours	After peroral administration of 1 4 ^{14}C spermidine				After peroral administration of 1 4 ^{14}C spermine			
	Radioactivity in spermidine cpm/total organ				Radioactivity in spermine cpm/total organ			
	Intestinal content	Intestinal tract	Liver	Other tissues	Intestinal content	Intestinal tract	Liver	Other tissues
1	230 000	150 000	15 000	9 800	340 000	140 000	1 000	440
3	260 000	130 000	31 000	17 000	310 000	46 000	2 000	1 000
5	330 000	73 000	19 000	16 000	140 000	24 000	1 400	1 100
8	190 000	79 000	22 000	11 000	150 000	34 000	1 400	1 100
16	3 300	18 000	15 000	10 000	5 800	2[illegible] 000	1 300	950

tract and its contents in the liver and the rest of the rat tissues was analysed 1 to 16 hours after a peroral treatment During the experiment the rats were fed otherwise *ad libitum* by a standard diet One month old rats were used The results as seen in Table IV revealed that both labelled polyamines can be absorbed from the intestinal tract The amounts of absorbed 1 4 ^{14}C spermidine and 1 4 ^{14}C-spermine differed a great deal from each other Around 6 per cent of the ^{14}C spermidine but only 0 4 per cent of the ^{14}C spermine was recovered after a peroral administration in the liver at the maximum point 3 hours About two-thirds of both the absorbed polyamines were found in this tissue It is difficult to define the amounts of the label in the intestinal wall although the washed intestine (intestine and colon) contained a relatively large part of the total radioactivity Nevertheless a part of it might represent contamination by the intestinal contents On the other hand 16 hours after peroral treatment the label in the intestinal wall was much greater than in the intestinal content This finding speaks in favour of a marked absorption also into the intestinal wall

There was no radioactivity in the brains of the rat or in the fetus of pregnant rats after intraperitoneal injections of 1 4 ^{14}C polyamines In these cases the experimental periods were 1 to 8 days However the brain and the fetal tissues contained great amounts of both polyamines There is obviously a barrier in the placental and brain membranes because there was polyamine radioactivity e g in the placenta after injection of both labelled amines

The *radioactivity in the blood* even soon after injection with 1 4 ^{14}C labelled polyamines was low as seen in Table V It was determined 5 15 30 60 and 180 min and 24 hours after the intraperitoneal injections In this case 0 2 μC (about 2 x 10 cpm) 1 4 ^{14}C polyamines were injected into one month old rats weighing about 80 g each 2 ml of the blood was taken by cardiac puncture and it was immediately injected into 4 ml of 0 1 N hydrochloric acid which was analysed using the routine method The highest values of injected polyamines in the blood were found already 5 min after injection Thereafter the polyamine radioactivity of the blood gradually decreased being a third 180 min after injection 24 hours later only traces of the label were detected in the blood

Table V 1,4 ^{14}C polyamines in the blood after an intraperitoneal injection 0.2 µC (2×10^5 cpm) 1,4 ^{14}C spermidine and spermine were injected into one month old rats _ rats at time were analysed 2 ml of blood was taken by cardiac puncture The results are presented as cpm/ml of blood

Time after injection	1 4 ^{14}C-polyamines in blood	
	After injection of 1 4 ^{14}C spermidine	After injection of 1 4 ^{14}C spermine
	Spermidine cpm/ml	Spermine cpm/ml
5 min	670	740
15 min	550	660
30 min	340	400
60 min	.60	270
180 min	210	240
24 hours	traces	traces

DISAPPEARANCE OF 1 4 ^{14}C POLYAMINES IN TISSUES

The elimination of the injected 1 4 ^{14}C polyamines will be presented as a function of time in some tissues At appropriate time intervals after injection the radioactivity of the given polyamine has been analysed in the liver kidneys and intestine The first analyses in these experiments were performed 3 hours after injection Both labelled spermidine and spermine were administered intraperitoneally The dose of radioactivity was 0 5 µC per 100 g rat weight about 5 000 cpm per g 1 1/2 months old rats were used

The elimination of 1 4 ^{14}C spermidine is presented in Fig 1 The values are shown as counts per min per gram of tissue (wet weight) It is seen that the radioactivity in spermidine declines rapidly in the kidneys and in the liver during the first 24 hours after the injection Later the decrease is gradually smaller The behaviour of the ^{14}C spermidine in the intestine is unexplained It does not actually seem to decrease

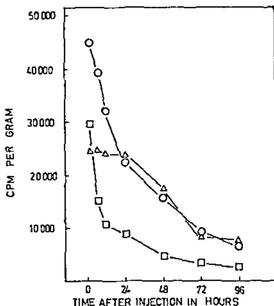


Fig 1 Di appearance of injected 1 4 ^{14}C spermidine in a few tissues 0 5 µC (about 5 x 10^{5} cpm) per 100 g rat weight of 1 4 ^{14}C spermidine was in jected intraperitoneally 3 to 96 hours after injection the radioactivity re maining in the spermidine was analysed The age of the rats was one month Mean values of 3 to 4 animals in one group are presented

○ *liver* □ *kidneys* Δ *intestine*

during the first 24 hours after the injection After this period the elimi nation is fairly similar to that in the other tissues

In addition it can be seen from Fig 1 that the radioactivity in all the e three tissues was about 6 to 10 times higher per gram of tissue at the beginning of the experiment than the dose of radioactivity was per gram of rat weight The 1 4 ^{14}C spermidine radioactivity per gram 3 hours after injection was about 46 000 in the liver about 30 000 in the kidneys and about 24 000 cpm/g in the intestine wherea the dose was only about 5 000 cpm/g of rat weight

In Fig 2 the elimination of 1 4 ^{14}C spermine is shown The same tissues have been analysed When the elimination of ^{14}C sper mine is compared to the corresponding elimination of ^{14}C spermidine some clear differences can be noticed The radioactivity of spermine decreases rather slowly in the liver and kidneys On the other hand the elimination of ^{14}C spermine seems in this case to be most rapid in the intestine These results show that the turnover rate of exogenously administered 1 4 ^{14}C-

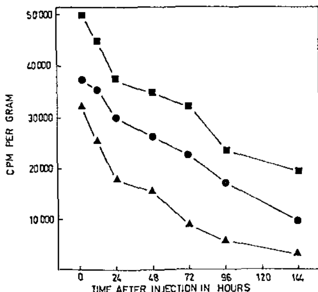


Fig 2 Disappearance of injected 1,4 ^{14}C spermine in a few tissues A comparable study to that presented in Fig 1 ● liver ■ kidneys ▲ intestine

spermine is two three times slower than that of spermidine in the liver and kidneys with rats aged 1 1/2 months

Again the radioactivity of 1 4 ^{14}C spermidine per gram was markedly higher in these tissues than in the average tissues In this case the activity of ^{14}C spermine per gram was at its highest level in the kidneys and at its lowest in the intestine throughout the experimental time as seen from Fig 2

The high values of the 1 4 ^{14}C spermine radioactivity in the kidneys agree with the nephrotoxic effect produced with this compound as observed by TABOR and ROSENTHAL (1956)

Experiments were not performed to study precisely the elimination of 1 4 ^{14}C-polyamines in rat at various ages However the evidence obtained suggests that there are no essential differences between the rates of disappearance of administered polyamines according to age Newborn rats were the only exception The total radioactivity in spermidine and spermine decreased markedly more slowly after injection of ^{14}C spermidine in new born rats during the four first day of life than in older rats

EXCRETION OF RADIOACTIVITY DERIVED FROM 1 4 ^{14}C-POLYAMINES

In this section the elimination of the label derived from 1 4 ^{14}C spermidine and 1 4 ^{14}C spermine into the urine and intestine as well as the degradation into carbon dioxide was studied after an intraperitoneal injection of these labelled amines Unfortunately only the fate of two labelled carbon atoms in these molecules could be investigated In fact the same carbon atoms were labelled both in spermidine and spermine the two at the ends of the 4 carbon chains which are derived from putrescine molecule

EXCRETION INTO URINE

It was expected that radioactive spermidine and spermine injected to rats would partly be excreted unchanged in the urine In fact ROSENTHAL and TABOR (1956) have shown that a part of the administered polyamines were excreted into the urine by injecting nonisotope polyamines to rats mice and rabbits However they used considerably higher quantities of these substances about 0 025–0 07 mmole per 100 g rat weight than used in this work The present experiments were performed to measure the amounts of labelled polyamines in the urine after administration of trace amounts of ^{14}C spermidine and ^{14}C spermine

The urine was collected daily after intraperitoneal injection of labelled polyamines The dose of radioactivity was 0 5 to 1 0 μC per 100 g rat weight Comparable results were obtained with these doses The injected radioactivity represented 0 75 to 1 49 μmoles of spermidine or 1 35 to 2 30 μmoles of spermine The method of urine collection has been described in the methods

The results reveal that neither labelled spermidine nor spermine was found in the urine after injection of traces of these amines However other unidentified radioactive compounds were excreted which yielded putrescine and polyamines during the strong acid hydrolysis The total radioactivity of the alkaline butanol soluble fraction of the urine has been demonstrated in Fig 3 during eight days after injection This fraction represents around 85 to 95 per cent of the total radioactivity in the urine The urinary radioactivities after injection of labelled spermidine and sper

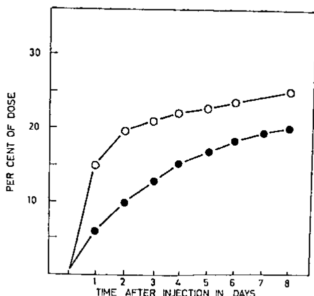


Fig 3 Excretion of radioactivity derived from injected 1 4 ^{14}C polyamines into urine

1 0 µC (about 10^6 cpm) 1 4 ^{14}C labelled permidine or spermine was injected intraperitoneally After injection the urine wa collected daily The alkaline butanol soluble radioactivity of the urine is demonstrated as a percentage of the dose The age of the rats was one month The radioactivity after injection of 1 4 ^{14}C spermidine ○ and 1 4 ^{14}C spermine ●

mine can be seen as a percentage of the daily dose in Fig 3 15 per cent of the given dose was excreted in the urine during the first day when ^{14}C spermidine had been administered After the fourth day the daily ex cretion represented only about one per cent of the dose The radioactivity in the urine derived from ^{14}C spermine was about 6 per cent during the first day It seems to rise more rapidly during the 2nd – 8th day than the radioactivity derived from ^{14}C spermidine

EXCRETION INTO INTESTINE

Polyamines can be converted into each other in rat tissues as will be shown later However a possibility is that the tissues themselves do not perform the interconversion of exogenously administered polyamines To try to exclude the possibility that intestinal microorganisms might be responsible for these reactions the intestinal content was analysed after intraperitoneal injections of the label

1 4 ^{14}C-polyamines were injected intraperitoneally The analyses were made 1 to 4 days after injection The radioactivity of the polyamines was determined from the total content of the washed intestine and colon which was *diluted with 0 1 N hydrochloric acid before the routine analysis* The animals were fed ad libitum during this experiment

In Table VI the total radioactivities of polyamines in the intestinal content are given when 0 5 uC (about 5×10^5 cpm) ^{14}C spermidine or ^{14}C spermine had been administered seperately Both labelled polyamines were discovered inside the intestine The values represent about one per cent of the dose per day During the second day the radioactivity of spermine increased to about double in the intestinal content after ^{14}C spermidine injection as did also the radioactivity of spermidine after a ^{14}C spermine administration

When the slight absorption of these amines after peroral treatment (Table IV) is kept in mind it is not obvious that the small amounts of formed polyamines in the intestinal contents could practically be responsible for the interconversion in tissues

No other alkaline butanol soluble compounds with the label could be detected in the acid intestinal content

Table VI The labelled polyamines in the intestinal contents after an injection of both 1 4 ^{14}C spermidine and spermine The intraperitoneal dose of radioactivity was 0 5 µC (5×10^5 cpm) per 100 g rat weight The total contents of the intestine and colon were analy ed 1 to 4 days after injection Means of 3 animals in a group are shown

Time after injection in days	Radioactivity in intestinal content (cpm/total)			
	After injection of 1 4 ^{14}C spermidine		After injection of 1 4 ^{14}C spermine	
	Spermidine	Spermine	Spermidine	Spermine
1	7 800	1 500	800	6 100
2	6 200	3 200	1 600	4 800
3	4 000	3 100	1 700	3 200
4	2 900	2 000	950	2 300

DEGRADATION TO CARBON DIOXIDE

When the recovery of the given radioactivity was studied the day after intraperitoneal injection of labelled polyamines a constant loss of radioactivity was observed which was more marked after the administration of ^{14}C-spermidine Attention was paid to the possibility that polyamines are degraded also to respiratory carbon dioxide

1 0 µC (about 10^6 cpm) 1 4 ^{14}C spermidine and spermine were injected intraperitoneally to the rats One to two month old rats were used In Table VII the mean values obtained during the first 12 hours after injection of the label are given The amounts of ^{14}C labelled carbon dioxide are expressed as a percentage of the injected radioactivity and as the total radioactivity at intervals of two hours Accordingly a part of the labelled polyamines is degraded to respiratory carbon dioxide About 8 per cent

Table VII Degradation of the label derived from 1,4 ^{14}C polyamines to respiratory CO_2

1 0 µC (about 10^6 cpm) of 1,4 ^{14}C spermidine and spermine was injected intraperitoneally The labelled CO_2 was collected from the outlet air for 12 hours after injection Means of 3 animals in a group are shown The total radioactivities in CO_2 and the radioactivities as a percentage of the injected dose are presented at time intervals of 2 hours

Time intervals after injection in hours	Radioactivity in carbon dioxide			
	After injection of 1 4 ^{14}C spermidine		After injection of 1 4 ^{14}C spermine	
	cpm/total CO_2	per cent of dose	cpm/total CO_2	per cent of dose
0 — 2	26 000	2 1	8 700	0 7
2 — 4	18 000	1 4	7 400	0 6
4 — 6	13 000	1 1	6 600	0 5
6 — 8	15 000	1 2	8 300	0 7
8 — 10	14 000	1 2	8 400	0 7
10 — 12	13 000	1 1	5 800	0 5
0 — 12	98 300	8 2	45 300	3 7

and RAINA 1966) and ornithine (RAIHÄ JÄNNE and SUIHKONEN 1967) are known to serve as precursors of spermidine and spermine in rat tissues According to the evidence from these spermidine is a precursor of spermine The present experiments were designed to confirm the synthesis of spermine from spermidine in rat tissues by the use of exogenous ^{14}C labelled spermidine as a precursor

In this study labelled spermidine was injected into rats intraperitoneally The dose of radioactivity was 0 5 μC (5×10^5 cpm) per 100 g rat weight The specific activity of the injected material was 6 6 mC/mmole This amount was small enough not to change the endogenous pool of polyamines essentially

At appropriate time intervals after injection the livers were analysed three to six at a time The radioactivity and amounts of liver polyamines were determined Fig 6 shows the specific activity of the formed spermine and remained spermidine (cpm/μmole) as a function of time in the liver The specific activity of spermine increased steadily for about four days after the 1 4^{14}C spermidine injection Then it began to decrease but the fall

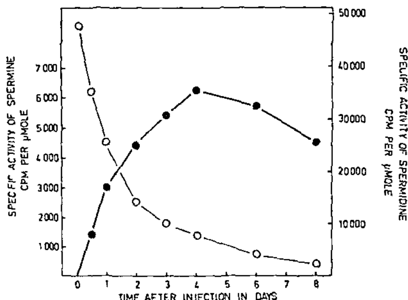


Fig 6 Formation of spermine from 1 4 ^{14}C spermidine in liver 0 5 μC (about 5×10^5 cpm) of 1,4 ^{14}C spermidine per 100 g rat weight was injected intraperitoneally The age of the rats was one month Mean values of 3 eto 6 animals in a group are demonstrated The specific activity of spermidine and spermine (cpm/μmole) are presented as a function of time
● spermine ○ spermidine

was fairly slow 8 days after the spermidine injection the spermine radioactivity was still at the level in the liver that it had been at 6 days earlier The radioactivity in the spermine exceeded that remaining in the spermidine in the liver only 8 days after the injection of labelled spermidine The changes in the polyamine contents during these 8 days due to age variations observed by JÄNNE RAINA and SIIMES (1964) were without any practical significance in this experiment

The spermine was synthesized to an equal degree in the liver when 1 4 ^{14}C-spermidine had been injected subcutaneously

Only poor incorporation into spermine was noticed *in vitro* with liver slices or liver homogenate

The methods of identifying the formed radioactivity of the electrophoretic fraction of spermine has been previously described in the methods

INCORPORATION OF 1 4 ^{14}C SPERMINE INTO SPERMIDINE IN LIVER

The incorporation of radioactivity from labelled spermidine into spermine was expected and had already been previously demonstrated by different precursors in the rat The converse case that administered 1 4 ^{14}C spermine led to the formation of spermidine was a somewhat unexpected

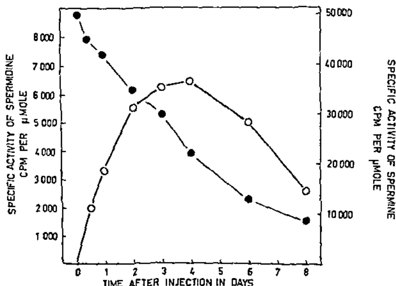


Fig 7 Formation of spermidine from 1 4-^{14}C spermine in liver
A compa able study to that represented in Fig 6 ○ spermidine ● spermine

observation in rat tissues In these experiments 0 5 μC (about 5×10^5 cpm) 1 4 ^{14}C labelled spermine (4 2 μC umole) per 100 g rat weight was injected intraperitoneally

First the incorporation into spermidine was studied at appropriate time intervals in the liver after an injection of labelled spermine Fig 7 demonstrates the specific activity of spermidine and spermine as a function of time The rate of degradation of 1 4 ^{14}C spermine to spermidine through the loss of a propylamine group is seen to be linear during the first two days after injection The maximum point in this reaction occurs at 3 to 4 days The specific activity of spermidine 8 days after the 1 4 ^{14}C-spermine administration was about half of the formed spermine in the converse reaction seen in Fig 6 This is the only essential difference when compared to the corresponding curve of the spermine formation These observations also confirm that the turnover rate of spermidine formed in cells is about twice as high as that of spermine

The experiments demonstrated in Fig 6 and 7 show that the reactions between exogenously administered polyamines are reversible in the rat liver

Table VIII *Formation of spermine from 1 4 ^{14}C spermidine in tissues at different ages 48 hours after injection with labelled spermidine*
The analyses were performed on newborn and 3 5 8 and 30 week old rats The dose of radioactivity was 0 5 μC per 100 g rat weight (about 5×10^5 cpm per g rat weight) in every age group Means of 3 to 4 animals in a group are shown except the newborn values which are means of 3 pooled samples The values have been presented as counts per min per gram of tissue (wet weight)

Age of rats in weeks	Formation of spermine (cpm/g)			
	Liver	Kidneys	Pancreas	Intestine
Newborns	1 400	3 000	—	1 900
3	1 600	2 200	1 300	2 500
5	2 900	3 000	2 400	4 600
8	4 100	2 600	2 300	4 300
30	3 800	4 600	[illegible]000	5 800

Here also only poor incorporation was observed *in vitro* with liver slices or liver homogenate

The purification and identification experiments of the formed spermidine radioactivity have been described in the methods

INTERCONVERSION OF 1 4 ^{14}C-POLYAMINES IN DIFFERENT TISSUES IN RELATION TO AGE

The polyamine contents in various rat tissues differ considerably from each other The molar ratio spermidine/spermine is unusually high in the rat pancreas and rather low in the kidneys (ROSENTHAL and TABOR 1956) In addition it has been observed that the concentration of spermidine decreases clearly with age per gram of tissue (wet weight) in the rat tissues The fall is greatest during the first month of life Because the changes in spermine contents are generally small the molar ratio of spermidine to spermine decreases in relation to age in every tissue studied (JÄNNE RAINA and SIIMES 1964)

In this section the appearance of radioactivity in spermidine and spermine after injection of 1 4 ^{14}C labelled polyamines is compared with age The liver kidneys pancreas and intestine were analysed The experiments were carried out with newborn rats and 3 5 8 and 30 weeks old rats In this case the analyses were performed only 48 hours after an intraperitoneal injection of labelled spermidine or spermine The dose of radioactivity was 0 5 μC (about 5×10^5 cpm) per 100 g rat weight in every age group

Table VIII represents an experiment showing the synthesis of spermine from ^{14}C spermidine in the liver kidneys pancreas and intestine The values are expressed as counts per min per gram of tissue (wet weight) It is difficult to compare the spermine synthesis in these tissues without simultaneously paying attention to the corresponding uptake *) of the spermidine in these tissues Accordingly Fig 8 shows the radioactivity in spermine as a percentage of the spermidine uptake 48 hours after injection of 1 4 ^{14}C spermidine It can be seen that the variation with age in the spermine synthesis from labelled spermidine is fairly small in every tissue studied Actually there are quantitative changes in the radioactivity of

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*) In this study the uptake of a polyamine is defined as the total radioactivity which can be found in a tissue at a certain time in this case 48 hours after a ^{14}C polyamine injection From practical point of view the uptake of one of the polyamines is the total radioactivity in spermidine and spermine because no other radioactive compounds were detected in the rat tissues as demonstrated in the methods

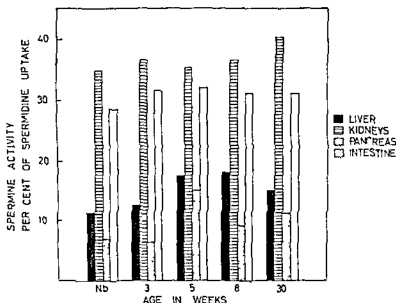


Fig 8 Percentage formation of spermine from 1 4 ¹⁴C spermidine in a few tissues in relation to age

Spermine radioactivity has been shown a a percentage of the permidine uptake (radioactivity in spermidine and spermine 48 hours after injection of 1,4 ¹⁴C spermidine) The details in Table VIII

spermine with age as seen in Table VIII but they are in proportion to the corresponding uptake of spermidine in these age groups as shown in Fig 8 The spermine synthesis as a percentage of the spermidine uptake

Table IX Concentrations and molar ratios of polyamines in a few tissues The age of the rats was 30 weeks Means and ranges of 8 animals in a group are shown

Tissue	Concentrations of polyamines: Spermidine mμmoles/g	Concentrations of polyamines: Spermine mμmoles/g	Molar ratio of spermidine to spermine
Liver	580 (545 615)	780 (742 — 836)	0 74 (0 69 — 0 82)
Kidneys	208 (198 214)	604 (583 — 638)	0 34 (0 32 — 0 35)
Pancreas	1 9-0 (1 680 2 050)	446 (417 — 466)	4 32 (4 72 — 4 00)
Intestine	760 (712 799)	540 (488 — 561)	1 40 (1 26 — 1 72)

is very similar throughout life especially in the kidneys and intestine However proportionally less spermine was formed in the liver from spermidine during the first two days of life than in the livers of animals aged 5 weeks In the present experiments the spermine concentration per liver gram (wet weight) was about twice as high as at the age of 5 weeks than newborn

Table IX represents the concentrations and molar ratios of spermidine and spermine in some tissues in rats at 30 weeks of age The polyamine concentrations of the liver and kidneys in relation to age were essentially similar to those reported previously by JÄNNE RAINA and SIIMES (1964) In the present study a similar decrease with age in the spermidine concentration was noticed in the pancreas as in other tissues The behaviour of the spermidine concentration of the intestine was different per gram from other analysed tissues with age In this organ there was practically no decrease in the spermidine concentration or in the molar ratio of spermidine to spermine in relation to age

Fig 8 illustrates also that in the age group of 30 weeks old rats around 40 per cent of the spermidine uptake in the kidneys and only 10 per cent in the pancreas was found as spermine 2 days after the 1 4 ^{14}C-spermidine injection The corresponding value in the liver was 15 per cent It is interesting to compare these values with the endogenous concentrations and with the molar ratios of the polyamines in these tissues The molar ratio of spermidine to spermine was as given in Table IX 4 3 in the pancreas and only 0 3 in the kidneys In the kidneys proportionally

Table X Formation of spermidine from 1 4 ^{14}C spermine in tissues at different ages 48 hours after injection with labelled spermine The comparable details are shown in Table VIII

Age of rats in weeks	Formation of spermidine (cpm/g)			
	Liver	Kidneys	Pancreas	Intestine
Newborns	– 200	1 700	—	900
3	5 300	4 500	5 400	2 000
5	5 100	3 500	5 200	3 400
8	4 200	3 000	3 800	2 700
30	5 000	4 300	3 200	3 600

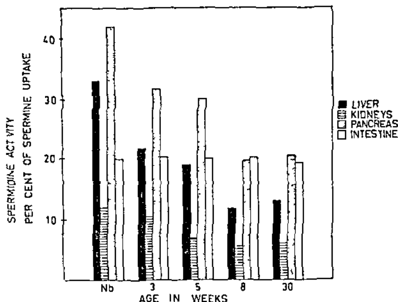


Fig 9 Percentage formation of spermidine from 1 4 ¹⁴C spermine in a few tissues in relation to age
Spermidine radioactivity has been shown as a percentage of the spermine uptake (radioactivity in spermine and spermidine 48 hours after injection 1 4 ¹⁴C spermine) The details in Table X

more spermidine was converted to spermine than in the pancreas The behaviour of the spermine synthesis in the intestine differed from the three other analysed tissues There was no similar correlation to the molar ratio of spermidine and spermine

Identical experiments were performed to study the converse reaction the incorporation of 1 4 ¹⁴C spermine into spermidine in relation to age Table X shows the radioactivity of spermidine per gram of tissue (wet weight) The formed radioactivity in spermidine per gram of liver is clearly higher in the young rats than the corresponding radioactivity in the spermine derived from spermidine which was shown in Table VIII On the other hand these liver values are similar when compared to each other in the older rats aged 8 or 30 weeks Analogous differences in the formation of spermidine and spermine can also be noticed in the kidneys and in the pancreas but not in the intestine

In Fig 9 the formed spermidine radioacticity is seen as a percentage of the spermine uptake This figure also shows that the conversion to spermidine is dependent on age The percentage values in young rats particularly newborn rats and those at 3 or 5 weeks of age are remark

ably higher in the liver pancreas and kidneys than those found in adult ones The incorporation into spermidine is also here similar in this case in the age groups of eight week and thirty week old rats

When the formation of spermidine in Fig 9 is compared in the tissues of adult rats some correlation to the molar ratio of spermidine to spermine can here be noticed also In the pancreas with a high molar ratio around 20 per cent and in the kidneys where the molar ratio is low, only 6 per cent of the spermine uptake is recovered in the spermidine during a period of 48 hours after the injection of 1 4 ^{14}C spermine The formation of spermidine in the intestine is very close to 20 per cent of the spermine uptake at every point of observation with age

INCORPORATION STUDIES WITH 1 4 ^{14}C POLYAMINES DURING LIVER REGENERATION

The formation of spermidine from some of its labelled precursors has been observed to be stimulated during liver regeneration The rise in the incorporation of 2 ^{14}C methionine into spermidine is about 10 fold during an incorporation period of 4 hours and it culminates at the point 12 to 16 hours after partial hepatectomy (RAINA JÄNNE and SIIMES 1966) The incorporation of 5 ^{14}C arginine (JÄNNE and RAINA 1966) and 1 4 ^{14}C-ornithine (JÄNNE under preparation) is also increased into spermidine On the other hand in a comparable study on the biosynthesis of spermidine from 1 4 ^{14}C putrescine somewhat surprising results were obtained by JÄNNE and RAINA (1966) They noticed that the specific activity of spermidine was clearly decreased 6 hours after partial hepatectomy after which the spermidine values again increased gradually to about control level In a preliminary experiment JÄNNE and RAINA (1966) also noticed that after injection of 5µC of 1 4 ^{14}C-spermidine at a point 22 hours post-operatively the liver total spermine radioactivity was $1\ 2 \times 10^5$ cpm one day after administration and $3\ 3 \times 10^5$ cpm 5 days after administration It was also observed that 5 days after injection the specific activity of spermine exceeded that of administered spermidine

The concentration of spermidine per unit weight has been shown to rise markedly in the liver during regeneration The increase in the concentration of this amine culminates at 64 to 72 hours after a partial hepatectomy DYKSTRA and HERBST 1965 RAINA JÄNNE and SIIMES 1965 1966) On the other hand the spermine concentration per liver gram decreases first and reaches its lowest values 32 hours afterwards where

after the concentration of spermine gradually increases but exceeds the control level only at a point 216 hours after operation

The present experiments were designed to find some further explanation for the behaviour of polyamines and for the role of interconversion reactions between exogenously administered spermidine and spermine during the regeneration

The technique used for partial hepatecotmy has been given in the methods The operation was performed 4 to 72 hours before decapitation The rats were injected at different times after partial hepatectomy and they were sacrificed simultaneou ly three hours after injection of the label It was necessary to find as short an incorporation time as possible to reflect the quick changes in the metabolism of polyamines after partial hepatectomy Incorporation times of one to 24 hours (SIIMES 1966) were tested but that of three hours was selected for these experiments During that time the changes in the radioactivities were large enough for reliable measurements Three hours was also supposed to be a time short enough

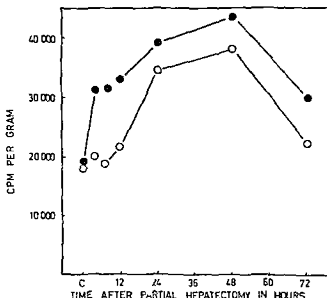


Fig 10 Uptake of 1,4 ¹⁴C polyamines in regenerating liver
Partial hepatectomy was performed 4 to 72 hours before decapitation Two separate experiments are presented one in which 0 2 μC (about 2×10^5 cpm) 1,4 ¹⁴C spermidine and another in which 0 2 μC (about 2×10^5 cpm) 1,4 ¹⁴C spermine was injected intraperitoneally 3 hours before analysis Mean values of 4 animals in a group are presented The age of the rats was one month
○ spermidine ● spermine

to reflect the changes in the polyamine metabolism The incorporation of radioactivity during this 3 hour period before decapitation was used as a measure of the degree of stimulation of the polyamine conversion 0 2 to 0 5 μC 1 4 ^{14}C labelled spermidine and spermine were separately injected intraperitoneally One-month-old rats were used

Uptake of administered 1 4 ^{14}C-polyamines First in Fig 10 the uptake of injected 1 4 ^{14}C spermidine and 1 4 ^{14}C spermine are seen in the regenerating liver 3 hours after injection The values are expressed as counts per min per gram of liver (wet weight) The uptake of ^{14}C spermine begins to rise soon after partial hepatectomy It is already nearly double 4 hours after the operation compared to the untreated controls The maximum point of spermine uptake is reached 48 hours after operation

The curve presenting the uptake of 1 4 ^{14}C spermidine in Fig 10 differs to some extent from that of 1 4 ^{14}C spermine There are no marked changes in the spermidine uptake at the time interval from 4 to 12 hours after partial hepatectomy At 24 and 48 hours the spermidine uptake per gram of liver is clearly increased

Fig 11 represents two separate experiments one in which the incorporation of radioactivity from 1 4 ^{14}C spermine into spermidine is de monstrated and another in which the incorporation of 1 4 ^{14}C spermidine both into spermine and into putrescine is shown during liver regeneration

Incorporation of 1 4 ^{14}C spermidine into spermine In this reaction only some slight changes seem to occur during the early period of regeneration at 4 to 12 hours after partial hepa tectomy as seen in Fig 11 At the later stages of the observation i e 24 and 48 hours there is a slight increase in the radioactivity of sper midine per gram culminating at 48 hours The changes in the uptake of ^{14}C spermidine (Fig 10) and in the synthesis of spermine from exogenous ^{14}C spermidine (Fig 11) are fairly similar Accordingly approxi mately equal parts of the uptaken radioactivity in the liver has been converted into spermine at every point of observation

Incorporation of 1 4 ^{14}C spermine into spermidine As seen in Fig 11 a marked stimulation is seen in this reaction during regeneration The activity of hepatic spermidine increased from the control value 720 cpm/g to 2 700 cpm/g at a point 8 hours after partial hepa tectomy Thereafter the spermidine radioactivity gradually decreased but the values still remained at a high level during the first 24 hours after

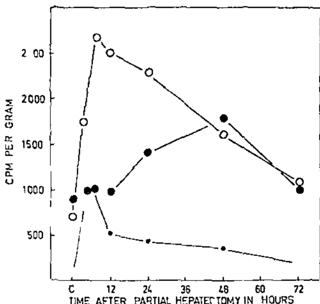


Fig 11 Formation of spermine ● and putrescine • from 1,4 ^{14}C spermidine and spermidine ○ from 1,4 ^{14}C spermine in regenerating liver
Partial hepatectomy was performed 4 to 72 hours before analysis 0 5 µC (about 5 x 10^5 cpm) of 1,4 ^{14}C labelled spermidine or spermine was injected intra peritoneally 3 hours before decapitation The age of the rats was one month Mean values of 4 animals in a group Cpm per gram of liver (wet weight) i demonstrated

operation The rise in the incorporation into spermidine seems to be quantitatively the most remarkable change of all the reactions studied during the liver regeneration In addition the specific activity of spermidine increased at 4 hours it was about 2 5 times and at 8 hours about 4 times the control values At the latest stages of observations the specific activity of the formed spermidine gradually decreased below the control values

Incorporation of 1 4 ^{14}C spermidine into putrescine It was surprising to notice that there was some radioactivity in the putrescine fraction after administration of ^{14}C-spermidine However by the standard method the radioactivity in the putrescine barely exceeded that of the background When the final concentration of the liver samples was 8-fold that of the routine one there was enough radioactivity that it could just be measured in the untreated livers The injection of labelled spermidine soon after partial hepatectomy caused a prompt increase in the radioactivity of putrescine At it was considerably increased, as revealed in Fig 11

An identical increase was obtained from 2 to 4 hours postoperatively when the incorporation time was only one hour The values at 2 hours were thus about half those at 4 hours As seen in Fig 11 the putrescine radioactivity was still increasing during the period from 4 to 6 hours The maximum values around 8 to 10 times the controls were found 6 hours after partial hepatectomy At an early moment after operation the concentration of putrescine per gram was increased about 2 to 3 times in the liver There was thus a smaller rise in the specific activity of the formed putrescine during the regeneration The experiments on the identification of the formed radioactivity in the putrescine fraction have been described in the methods

These experiments reveal that the conversion of exogenous spermidine to putrescine is possible in the rat liver This is worthy of note when the metabolism of polyamines is being discussed

I n c o r p o r a t i o n o f 1 4 ^{14}C-s p e r m i n e i n t o p u t r e s c i n e No radioactivity could be demonstrated in the putrescine fraction either in the control or hepatectomized liver samples after injection of 1 4 ^{14}C spermine during the regeneration

I n c o r p o r a t i o n e x p e r i m e n t s i n v i t r o The studies on the interconversion of labelled polyamines *i n v i t r o* with liver homogenate obtained from the livers 16 and 48 hours after partial hepatectomy were not successful

C o n c e n t r a t i o n s o f p o l y a m i n e s The changes in the concentrations of polyamines in the regenerating rat liver in the present study were comparable to those which have been reported previously by RAINA JÄNNE and SIIMES (1965 1966)

INTERCONVERSION OF 1 4 ^{14}C POLYAMINES DURING ETHIONINE TREATMENT

Methionine through the formation of S adenosylmethionine is a source of the three carbon moieties of spermidine and spermine in polyamine biosynthesis After administration of ethionine to rats large amounts of S-adenosylethionine are found in the liver (MODY BULBA HOLOWECKY and STEKOL 1963 RAINA JÄNNE and SIIMES 1964 SHULL McCONOMY VOGT CASTILLO and FARBER 1966) This compound is obviously unavailable for polyamine synthesis although no experimental evidence supports this conclusion Although many changes in tissue metabolism arise from ethionine administration most of them inhibitory to protein synthesis (STEKOL 1963) this compound also causes characteristic

changes in the polyamine concentrations during continous daily administration over several days first a decrease later a considerable rise in the spermidine and a fall in the spermine concentration per gram of rat liver (wet weight) (RAINA JANNE and SIIMES 1964 KREMZNER and STARR 1966) After a single injection of ethionine variable changes in the polyamine concentrations have been reported in the rat liver (RAINA JÄNNE and SIIMES 1964 SHULL McCONOMY, VOGT CASTILLO and FARBER 1966 NEISCH 1967)

In the following experiments DL ethionine was injected daily intraperitoneally for six days before decapitation The dose of ethionine was 0 25 or 0 6 mmole per 100 g rat weight per day A single dose of 1 4 ^{14}C spermidine 0 5 µC (5 x 10^{5} cpm) per 100 g rat weight was also injected intraperitoneally 1 to 4 days before decapitation One-month old rats were used

It can be seen from Fig 12 that the radioactivity left in spermidine was slightly although unsignificantly higher during ethionine administration compared to the controls Ethionine had practically no effect on the rate of elimination of the injected ^{14}C spermidine in the liver The degradation products of spermidine during ethionine treatment are not known Only the pathway to putrescine was studied but it was not activated

In Fig 12 the incorporation of radioactivity into spermine as a function of time in the livers of the control and ethionine treated groups are shown too The counts per total liver are presented 1 to 4 days after injection with 1 4 ^{14}C spermidine In this experiment the dose of ethionine was 0 25 mmole per 100 g per day but essentially comparable results were obtained during the daily dosage of 0 6 mmole Fig 12 shows that a clear inhibition is induced in the spermine synthesis from 1 4 ^{14}C spermidine by daily ethionine administrations

It takes about two days before the inhibitory effect on the spermine synthesis is fully developed When the ethionine treatment was interrupted two days before decapitation under otherwise identical experimental conditions as shown in Fig 12 the spermine synthesis resumed to some extent 1 to 2 days after interruption

A comparable study on the formation of spermidine from 1 4 ^{14}C spermine during ethionine treatment was also performed Daily ethionine injections 0 3 mmole per 100 g rat weight per

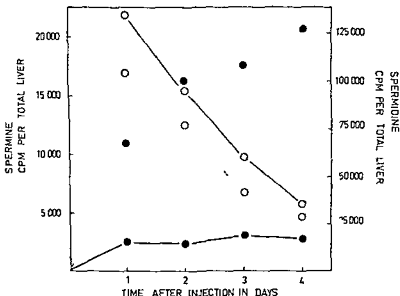


Fig 12. Effect of ethionine treatment on the elimination of injected 1,4 ^{14}C spermidine and on the synthesis of spermine in liver

DL ethionine (0.5 mmole/100 g rat weight) was injected intraperitoneally and daily for 6 days before decapitation A single injection of 1,4 ^{14}C spermidine (0.5 μC about 5×10^5 cpm) was also injected intraperitoenally 1 to 4 days before decapitation The age of the rats was one month Mean values of 4 animals in a group are presented The radioactivity in spermidine and spermine per total liver is shown ○ spermidine ● spermine —— ethionine treated — — — controls

day had no effect on the appearance of radioactivity in spermidine in the liver when compared with the untreated livers

CHANGES IN LIVER POLYAMINES DURING STARVATION

During the ethionine treatment the weight of the rats fell considerably depending on the dose of ethionine The rats were fed ad libitum but their food intake was quite small To eliminate the effects of starvation as a cause for polyamine changes during ethionine administration some experiments were carried out on rats fed only with water for 1 to 4 days Rats aged one month were used

The concentrations of polyamines per gram liver and per total liver (wet weight) are shown in Fig 13 The spermidine concentration of the liver gram began to fall only after a 3-day period of starvation On the 4th day the concentration of spermidine per gram was already nearly half that of the control values The liver spermine concentration per gram rose about 20 per cent ($p < 0.1$) during the first

DISCUSSION

M e t a b o l i s m o f p o l y a m i n e s i n t h e r a t Methionine putrescine arginine and ornithine (RAINA 1964 JÄNNE and RAINA 1966 JÄNNE under preparation) have been reported to be precussors of spermidine in the rat Arginine and ornithine are shown to be precursors in the biosynthesis of putrescine also in the rat liver (JÄNNE under preparation) Putrescine has also been demonstrated to be incorporated into spermidine in microorganisms (TABOR ROSENTHAL and TABOR 1956 1958) in Ehrlich ascites cells (SIIMES and JÄNNE 1967) and in the developing chick embryo (RAINA 1962 b 1963) The radioactivity in putrescine is also incorporated into spermine but much more slowly than into spermidine This slow incorporation into spermine gives cause to believe that spermidine is a precursor of spermine (RAINA 1963) Further support for this idea has been obtained by RAINA (1963) in experiments with developing chick embryo After administration of labelled methionine which is the source of the 3 carbon chains of polyamines radioactivity was incorporated simultaneously into both polyamines in roughly equivalent amounts These experiments also provided evidence that both propylamine moieties of spermine are derived from methionine On the other hand it has been pointed out that i n v i t r o systems spermidine could not form a substitute for putrescine as an acceptor of the propylamine moiety (TABOR 1962 b)

The present experiments as well as the preminminary experiments from this laboratory (JÄNNE and RAINA 1966 SIIMES 1966 1967) demonstrated by use of exogenous spermidine that spermidine is a precursor of spermine in rat tissues No evidence for some other metabolic route leading to the formation of spermine other than through spermidine has been presented Evidence that spermine can be converted into spermidine has been obtained in micro-organisms (RAZIN BACHRACH and GERY 1958 RAZIN GERY and BACHRACH 1959 BACHRACH PERSKY and RAZIN 1960) Further the degradation of spermidine to putrescine has been reported in P s e u d o m o n a s a e r u g i n o s a (PADMANABHAN and KIM 1965) The reversible interconversion between putrescine spermidine

and spermine in a single organism has not previously observed although the reactions have been separately demonstrated in different organisms The present work shows that exogenous spermidine and spermine can be converted into each other and that spermidine can also be degraded to putrescine in rat liver These observations indicate that there are reversible reactions between putrescine and polyamines in the rat

In addition to the interconversion reactions the polyamines can be metabolized through other routes in rat tissues This area of polyamine metabolism was not studied in detail in the present work However it was noticed that a considerable portion of radioactivity was found as CO derived from the carbon 1 and 4 in spermidine and spermine The metabolic routes of this degradation are not known The formation of carbon dioxide from 1 4 ^{14}C spermine was considerably smaller than that from 1 4 ^{14}C spermidine under the same experimental conditions It is possible that the degradation of spermine might go through spermidine to CO_2

Several radioactive metabolites of polyamines and putrescine were found in the alkaline butanol soluble extract of the urine during a period of 8 days after injection with 1 4 ^{14}C polyamine These compounds were not identified in this study Similar radioactive compounds were not found however in the liver 24 or 48 hours after injection It was interesting to note that the strong acid hydrolysis of the acid urine yielded large amount of putrescine in addition to polyamines In other experiments putrescine was found to be a degradation product of 1 4 ^{14}C spermidine in regenerating liver but not in the normal liver The studies on the urine indicate that the untreated animal might to a large extent also have the ability degrade spermidine to putrescine which would be excreted into the urine as a conjugate e g as acetylputrescine

The experiments with the chick embryo indicated the slow elimination of endogenous polyamines (RAINA 1963) It was also obvious that the turnover rate of spermine was slower than that of spermidine Similar findings were also obtained in the present experiments The elimination of both the exogenous spermine and that formed in the cells was clearly slower than that of exogenous spermidine For instance in the liver 8 days after the 1 4 ^{14}C spermidine injection the amount of radioactive spermine exceeded that of spermidine The slower degradation to CO and the slower excretion of radioactivity into the urine after injection with ^{14}C-spermine also indicate a slower turnover rate in spermine than in spermidine

It has been observed that E coli (TABOR and TABOR 1964a 1966) and Ehrlich ascites cells (JÄNNE and SIIMES, under prepation) can as well take up putrescine and polyamines as synthesize these compounds Naturally the present results concerning the uptake of labelled polyamines do not define the localization of the label in the cells According to the studies with E coli and Ehrlich ascites cells it is obvious that a part of the label in the medium is adsorbed on the cells and a part is transported into the cells It is likely that a major part of the shown uptake is intracellular as late as 48 hours after injection of a labelled polyamine

The importance in animals between the exogenous and the polyamines synthesized in the tissues is not known The present results revealed that part of the polyamines and particularly of the spermidine can be absorbed from the intestine after peroral treatment Rats have also been fed on a diet which lacked polyamines (ROSENTHAL and TABOR 1956) This treatment caused only a slight decrease in the liver concentration of spermidine A possibility might also be that the intestinal micro-organisms synthesize polyamines which are then partly absorbed from the intestine This question has been studied with the germ free rats of a third generation by ROSENTHAL and TABOR (1956) However, no large deviations were noticed in the liver concentrations of polyamines These observations suggest the importance of the biosynthesis of polyamines in rat tissues instead of the absorption from the intestine

Metabolism of polyamines during starvation During starvation the liver contents of the polyamines decreased clearly that of spermidine somewhat sooner than spermine per total liver It is likely that during starvation there is a deficiency of the essential precursor e g methionine needed in the biosynthesis of polyamines In fact the concentration of S adenosylmethionine has been reported to be nearly half of that of the normal liver value even after starvation lasting only one day (SCHLENK 1965) This might be a reason for the changes in polyamine concentrations during starvation Because of the slower turnover rate of spermine the decrease in the concentrations would first act on the spermidine As a result the molar ratio of spermidine to spermine would lessen during starvation The lack of spermidine is thus more intensive than that of spermine This would explain the increased degradation of 1 4 ^{14}C spermine to spermidine in the starved livers

Polyamine interconversion in relation to age

The varying concentrations of polyamines in different tissues and the fall in the tissue spermidine concentration with age (JÄNNE, RAINA and SIIMES 1964) are of interest In the present study some attempts were made to study the interconversion of polyamines in relation to age The clearest way to study these reactions in the tissues according to age would have obviously been the incorporation studies *in vitro* e g with liver slices or homogenate obtained from the rats of different ages Unfortunately these amines did not incorporate into each other under such conditions although the interconversion has been demonstrated in micro organisms (TABOR and TABOR 1964 b) and in Ehrlich ascites cells (SIIMES and JÄNNE 1967)

It is obvious that the interconversion between spermidine and spermine is partly responsible for the changes in polyamine concentrations with age The results showed that proportionally more of the exogenous spermine taken up in a tissue was converted into spermidine if the molar ratio of endogenous spermidine to spermine was high On the other hand the formation of spermine from spermidine was more active in tissues with a low molar ratio The mode of expressing the formation of polyamines from each other used here is questionable in the tissues of new born rats because the elimination of the exogenous spermidine given was slower in the tissues at least during the first four days of life However the rate of elimination of exogenous spermidine in the 3—30 week age group and that of exogenous spermine in every age group was found to be essentially similar in the tissues analysed In young rats aged 3 to 5 weeks more spermidine was clearly formed from exogenous spermine than spermine from exogenous spermidine during the 48 hours after injection of the label from an equal dose of a given polyamine This was true in the liver kidneys and pancreas but not in the intestine In addition when the whole animal was analysed more spermidine was formed in those age groups In the tissues mentioned there were practically no difference in the polyamine formation among the rats aged 8 to 30 weeks These results suggest that the formation of spermidine from labelled spermine is more active in young rats than in older ones

The above mentioned findings prompted a study of the metabolism of polyamines in a rapidly growing system which might give more quantitative data than the experiments relying on growth with age Regenerating rat liver was chosen for these experiments

Polyamine metabolism in regenerating liver and relation to nucleic acids The blood flow through the two liver lobes remaining after partial hepatectomy is increased during the 3 first hours after operation (MENYHART and SIMON 1966) LIEBERMAN, KANE and SHORT (1965) reported an interesting study in which they had experimentally investigated the effect of portal vein volume on the synthesis of RNA in the liver They noticed that rapid portal injections of a variety of solutions e g sodium chloride caused increases in the specific activities of RNA-polymerase and in the rates of the RNA synthesis These rises were indistinguishable from those obtained after partial hepatectomy Several changes in the formation of putrescine spermidine and spermine were noticed in this work during the liver regeneration It is difficult to exclude the possibilty that these changes would be partly the result of the increased blood flow through the regenerating lobes of the liver On the other hand the concentrations of radioactive polyamines in the blood were fairly low even soon after the intraperitoneal injections but nothing is known about the effectiveness of the liver cells in clearing polyamines from the blood If the clearance is intensive the increased blood flow obviously also increases the uptake of labelled polyamines during liver regeneration

Putrescine synthesis from its labelled exogenous precursors such as 5 ^{14}C-ornithine and 5 ^{14}C arginine has been shown to have risen even 2 hours after partial hepatectomy and reached its highest values at around 8 hours (RÄIHÄ JÄNNE and SUIHKONEN JÄNNE under preparation) The conversion of exogenous spermidine to putrescine is activated 2 to 6 hours after partial hepatectomy as shown in the present work However during the early stage of regeneration at 6 hours the incorporation of radioactivity from exogenous putrescine into spermidine was decreased according to JÄNNE and RAINA (1966) Earlier DYKSTRA and HERBST (1965) noticed an increase in the incorporation of exogenous putrescine into spermidine but these two studies are obviously not comparable to each other There were essential differences between these experiments e g in the route of administration of the label The maximun concentration of putrescine can be found at around 4 to 8 hours after operation At that time it is about 3 fold that of the controls The mentioned changes in the metabolism of putrescine i e its increa ed synthesis from 5-^{14}C-ornithine and arginine as well as the increased formation from 1 4 ^{14}C-spermidine and its possibly decreased incorporation into spermidine provide a good explanation for its accumulation during the early stage of liver regeneration

The synthesis of spermidine is also increased after partial hepatectomy culminating at 8 to 20 hours after operation This is seen through the incorporation of radioactivity into spermidine from labelled methionine (RAINA JÄNNE and SIIMES 1965, 1966) arginine (JÄNNE and RAINA 1966) and spermine The rise in spermidine radioactivity derived from exogenous spermine obviously begins immediately after partial hepatectomy and the highest values are seen at 8 hours The formation of spermidine from 1 4 ^{14}C spermine remained at a high level during the first 24 hours after operation The incorporation of radioactivity from 2 ^{14}C methionine into spermidine also rose immediately and culminated at 12 to 16 hours after partial hepatectomy (RAINA JÄNNE and SIIMES 1965 1966) In addition the incorporation of exogenous putrescine into spermidine which was reported to have decreased at 6 hours, rose to the control level during the interval from 6 to 16 hours after operation (JÄNNE and RAINA 1966) Accordingly the stimulation in the spermidine synthesis seems to occur particularly during the first 24 hours after partial hepatectomy As recently reported the liver spermidine concentration per weight unit begins to rise at a point 16 hours after operation It reaches its highest values at 64 to 72 hours (DYKSTRA and HERBST 1965 RAINA JÄNNE and SIIMES 1965 1966)

A lag period during the first 10 days of life has been noticed in the rat liver during the postnatal growth During this period liver weight does not increase (OLIVER BALLERD SHIELD and BENTLEY 1962) The liver of 3 week old animals grows rapidly and might be comparable to a regenerating liver In age groups of 3 to 5 weeks the formation of spermidine from exogenous spermine was the prominent feature in this reversible reaction as was also shown during the liver regeneration

In contrast no noteworthy changes could be found in the spermine synthesis from exogenous spermidine at 4 to 12 hours after partial hepatectomy At that time there was practically no stimulation either in the incorporation into spermine from exogenous methionine (RAINA JÄNNE and SIIMES 1965 1966) although in the untreated livers this precursor was incorporated into both polyamines in roughly equivalent amounts (RAINA 1964) In the light of the above findings the activated degradation of exogenous spermine to spermidine at the early stage of regeneration might lead to a fall in the concentration of spermine per gram of liver (wet weight) seen during the period of 16 to 32 hours (RAINA JÄNNE and SIIMES 1965 1966) The rise in the incorporation of radioactivity from 1 4 ^{14}C spermidine to spermine was found to occur at a fairly late stage

after operation i e at 24 and 48 hours after partial hepatectomy The total contents of spermine per residual liver is slowly increasing in the regenerating liver As late as 96 to 216 hours after operation the spermine contents have clearly exceeded the control level (RAINA JÄNNE and SIIMES 1966)

A phasic increase in the concentrations of putrescine spermidine and spermine is obvious during liver regeneration The rise in liver putrescine culminates at 4 to 8 hours and that of spermidine around 64 to 72 hours and the concentration of spermine is still slowly increasing 216 hours after partial hepatectomy On the other hand the concentrations of most free amino acids and related compounds in the liver stay unchanged at 6 and 24 hours postoperatively (FERRARI and HARKNESS 1954) except those of aspartic acid glutamic acid and lysine which have been shown to be clearly elevated 6 hours after partial hepatectomy (FERRARI and HARKNESS 1954) A less marked increase in the above-mentioned amino acids was noticed at 24 hours The concentration of glutamine has been reported to be decreased in regenerating rat liver (CHRISTENSEN, ROTH WELL SEARS and STREICHER 1948 FERRARI and HARKNESS 1954)

During the last few years evidence has been accumulated on the relation of polyamines to nucleic acids A very close correlation between the concentrations of total polyamine nitrogen and RNA phosphate has been observed during the exponential growth of E coli by RAINA and COHEN (1966) and by COHEN HOFFNER JANSEN MOORE and RAI NA (1967) These correlations are interesting because it has been noticed a stimulatory effect by polyamines and putrescine on DNA primed RNA polymerase activity in vitro (KRAKOW 1963, FOX ROBINSON, HASELKORN and WEISS 1964 FOX and WEISS 1964) and on the DNA polymerase (BREWER and RUSCH 1966) In a polyauxotrophic strain of E coli RAINA and COHEN (1966) reported that a relatively high concentration of exogenous spermidine caused a marked rise in the uracil incorporation into RNA during amino acid starvation An increase in RNA synthesis has also been obtained by spermine in E coli (MILLS and DUBIN 1966) and in a tissue culture (GOLDSTEIN 1965)

In animal tissues correlations between the changes in the concentrations of nucleic acids and polyamines particularly between those of RNA and spermidine have also been noticed e g during the embryonic development (RAINA 1963 CALDARERA BARBIROLI and MORUZZI 1965) in the rat liver during ionizing radiation (CALDARERA COZZANI and MO

RUZZI 1966) and during growth hormone treatment (JÄNNE 1967) and especially in the intracellular distribution of RNA and spermidine (RAINA and TELARANTA 1967)

The changes in the concentrations of RNA and spermidine have been shown to be fairly similar during liver regeneration (DYKSTRA and HERBST 1965 RAINA JÄNNE and SIIMES 1965 1966) On the other hand a few hours after partial hepatectomy there is a rise in the synthesis of RNA reflected in the increased incorporation of labelled orotic acid (HECHT and POTTER 1958 FUJIOKA KOGA and LIEBERMAN 1963 UCHIYAMA FAUSTO and LANCKER 1966) orthophosphate (NYGAARD and RUSCH 1955 WELLING and COHEN 1960) and glucose (SCHNEIDER and POTTER 1957) into RNA The RNA polymerase activity has also been reported to increased in the liver after partial hepatectomy A certain rise has been noticed even at 2 to 6 hours The maximum values have been found 12 to 24 hours after operation (BUSCH CHAMBON MANDEL and WEILL 1962 TSUKADA and LIEBERMAN 1964 RO and BUSCH 1967) In contrast a rise in the incorporporation into DNA occurs later 15 to 18 hours after partial hepatectomy (NYGAARD and RUSCH 1955 HECHT and POTTER 1956 1958 SCHNEIDER and POTTER 1957 WELLING and COHEN 1960)

It is obvious that many changes occur in the metabolism of polyamines and putrescine before the tissue actually begins to grow In the regenerating liver the formation of putrescine and spermidine are active at the same time as the stimulation in the synthesis of RNA

Polyamine metabolism in relation to ethionine treatment Ethionine is a toxic compound whose effects on tissues are obviously based on the antagonism of methionine (STEKOL 1963) During ethionine treatment relatively large amounts of S adenosylethionine are found in the rat liver (MODY BULBA HOLOWECKY and STEKOL 1963 RAINA JÄNNE and SIIMES 1964 SHULL MCCONOMY VOGT CASTILLO and FARBER 1966) The formed compound is obviously inactive as a substrate for the biosynthesis of polyamines for which S adenosyl methionine is an essential precursor (TABOR and TABOR 1964 b)

The effect of ethionine is dependent upon the daily dose the cessation of treatment and the tissue which has been analysed in the rat (SIIMES RAINA and JÄNNE under preparation) The day after a single injection of ethionine variable effects have been reported on the concentration of spermidine 0 1 mmole has caused a rise (NEISCH 1967) and 0 6 mmole

a fall (RAINA JÄNNE and SIIMES 1964 KREMZNER and STARR 1966) in the concentration of spermidine in the rat liver With 0 2 mmole of ethionine no effect was obtained on the spermidine concentration (RAINA JÄNNE and SIIMES 1964) On the other hand during daily ethionine treatment (0 6 mmole/100 g rat weight/day) there is first a fall and later a considerable rise in the concentration of spermidine (RAINA JÄNNE and SIIMES 1964 KREMZNER and STARR 1966) and a marked fall in the concentration of spermine of the rat liver (RAINA JÄNNE and SIIMES 1964) As a consequence of these changes the molar ratio of spermidine to spermine rose markedly in the liver

RAINA (1963) in the developing chick embryo showed that the rate of the synthesis of spermidine from ^{14}C methionine was not altered by a single injection of ethionine but that this treatment caused a decrease in the incorporation of ^{14}C methionine into spermine In the present experiments a single injection of ethionine produced only a slight decrease in the incorporation of exogenous spermidine into spermine in the rat liver However by the daily administrations of ethionine nearly the total inhibition originated in spermine synthesis from 1 4 ^{14}C spermidine

An explanation for the rise in spermidine and the fall in spermine concentrations during continuous treatment of ethionine might be the inhibition in the formation of spermine from spermidine The rise in spermidine might also be due to an increase in its synthesis As shown in the present experiments the formation of spermidine from exogenous spermine was not altered under these conditions JÄNNE (under preparation) has found that the synthesis of spermidine from exogenous putrescine is not increased during ethionine administration either

During the administration of ethionine the rats are practically starved because the intake of food is very poor during this treatment During the starvation without ethionine converse changes took place in the liver polyamine concentrations to those during ethionine treatment These findings suggest that relative starvation during administration of ethionine is not the only reason at least for the changes in the metabolism of polyamines

SUMMARY

The metabolism of exogenous tetramethylene 1 4-^{14}C-labelled poly amines spermidine and spermine was studied in the rat

1 Spermidine is a precursor in the biosynthesis of spermine in rat tissues The synthesis of spermine was most intensive in the organs where the molar ratio of spermidine to spermine was the lowest The converse reaction the degradation of exogenous spermine to spermidine was also demonstrated This conversion was marked in tissues with a high molar ratio The formation of spermidine from exogenous spermine was more prominent at the age of 3 and 5 weeks in the liver kidneys and pancreas than conversery but there was practically no difference at the age of 8 and 30 weeks in this reversible reaction

2 In the liver and kidneys the elimination of exogenous spermine was slower than that of spermidine In the liver the spermine synthesized from exogenous spermidine was also eliminated more slowly than the spermidine formed from labelled spermine 8 per cent of the spermidine and 4 per cent of the spermine radioactivity was degraded to respiratory CO during the first 12 hours after intraperitoneal injection 7 per cent of the administered radioactivity derived from ^{14}C-spermidine and 2 per cent of that from ^{14}C-spermine was excreted into the urine under similar experimental conditions The labelled compounds in the urine were not identified although they gave free putrescine and polyamines with the label during acid hydrolysis No free labelled polyamines could be found in the unhydrolyzed urine

3 A part of the perorally administered 1 4 ^{14}C spermidine and 1 4 ^{14}C spermine could be absorbed from the intestine About 6 per cent of the spermidine was maximally found in the liver but only 0 4 per cent when ^{14}C spermine was given *per os*

4 In the regenerating liver the incorporation of radioactivity from 1 4 ^{14}C-spermidine to putrescine was observed to be active at 2 to 6 hours after partial hepatectomy The formation of spermidine from 1 4 ^{14}C spermine was also increased immediately and markedly after operation The highest values were obtained at 8 to 24 hours The incorporation of 1 4 ^{14}C

spermidine to spermine did not rise at 4 to 12 hours In this reaction increased values were noticeable 24 and 48 hours after partial hepatectomy

5 Daily administration of ethionine caused nearly complete inhibition on spermine synthesis from 1 4 ^{14}C spermidine This treatment had no effect on the converse reaction from 1 4 ^{14}C spermine to spermidine

6 Starvation of 1 to 4 days caused a decrease in the spermidine and a slight increase in the spermine concentration Starvation increased the degradation of 1 4 ^{14}C spermine to spermidine but had no effect on the converse reaction

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ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 299

Carl Koller and the Development of Local Anesthesia

BY

G LILJESTRAND

STOCKHOLM 1967

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From the Department of Pharmacology The Caroline Institute
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Carl Koller and the Development of Local Anesthesia

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In 1963 in collaboration with Dr. now Professor B. Holmstedt, the author published a book entitled "Readings in Pharmacology" (*28*). It was an attempt to give a brief survey of the development of pharmacology from ancient times to the present day by direct quotations from more or less fundamental publications (translated into English when necessary). They were preceded by brief biographical notes on the scientists concerned and joined together so as to cover the more important fields. One of the twelve chapters dealt with local anesthesia and it contained among other things a considerable part of the lecture given on that subject especially with regard to the eye by the young Austrian physician Carl Koller (1857—1944) on October 17 1884 before the Wiener medizinische Gesellschaft (Vienna Medical Society). This work aroused in the author a lively interest in Koller his work and his life. I accidentally met one of my earlier fellow professors at the Caroline Institute Dr. J. Wilhelm Nordenson M.D. D.Sc. (1883—1965) at the time Emeritus Professor of Ophthalmology. I knew that he had met Koller in New York (*53*) and he told me a great deal about that meeting.

My colleague had obtained a considerable part of his scientific training with Allvar Gullstrand (1862—1930) the first professor of Ophthalmology at the University of Uppsala (*54*) wellknown for his many important contributions to our knowledge of the functions of the eye as well as numerous practical applications. In 1911 he was awarded the Nobel Prize in Physiology or Medicine for his work on the dioptrics of the eye. Three years later he was appointed to a personal professorship in Physical and Physiological Optics at the University of Uppsala which he held until he became Emeritus in 1927. The ordinary chair of Ophthalmology became occupied in 1915 by C. F. Lindahl M.D. (1874—1943) a chronic disease however compelled him to take temporary leave in the course of 1922 and Nordenson was charged to act as his substitute. The health of Professor Lindahl deteriorated however and in 1927 he retired completely and at the end of that year Nordenson became his successor. Soon afterwards (in 1931) he accepted a call to the corresponding chair at the Caroline Institute in Stockholm. In 1948 he retired having reached the age limit.

This paper is published in connection with the 50th anniversary of the author's rece
the M.D. degree at the Caroline Institute



Carl Koller in 1885
(After E Lesky)

Contact with American medicine had been practically impossible during the First World War and it took several years before it became normal again Professor Gullstrand was interested in making his own work known on the other side of the Atlantic and in studying the progress made there He also planned to try to pave the way for the renewal of international cooperation in his science consequently he attended as an official delegate of Sweden the International Congress of Ophthalmology (23) which took place in Washington D C on April 25—28 1922 He was accompanied by his former assistant Nordenson On April 25 at the Congress Gullstrand gave a much appreciated communication entitled On diaphragm lamps in Ophthalmology where he described the slit lamp and the aspheric lens as well as the hole or diaphragmatic lamp He also discussed focal illumination and several kinds of reflex free ophthalmoscopy from the large instrument with stereoscopic vision to the simplified one as well as the centric photography of the fundus oculi (23 69–80) Next day Professor Gullstrand gave a demonstration of Diaphragm Illumination and Dr Nordenson another about Centric Photography of the Fundus Oculi (23 695) According to Nordenson s account it might have been in this connection that he first met Koller With his customary civility Nordenson was introducing himself to those colleagues attending the demonstrations when he suddenly heard the name of Koller A collection



The m ddle aged Koller

of letters from Koller to Erik Nordenson (1847—1919) M D a well known ophthalmologist and the father of Wilhelm who inherited those letters had made him well acquainted with Koller s work so that contact rapidly became established Koller s name appears on the membership list of the Congress (*23* 19) but without the star indicating his presence so it is possible that he only attended the demonstrations given elsewhere and he may even have gone in order to meet Nordenson Since Nordenson in his biography of Gullstrand (*54* 329) tells us that the demonstrations by Gullstrand and Nordenson were also given in New York before a selected group at a session arranged by the local committee somewhat earlier than the Congress itself it might have been in that connection that he saw Koller It is certain however that a meeting between the two took place in New York This was mentioned in a speech by Nordenson which he delivered as Dean of the medial faculty at Uppsala on May 30 1931 (*53*) when conferring the degree of M D In commemoration of the first similar conferment in Sweden 250 years earlier, he then gave a lecture about the work of Koller on cocaine and mentioned his visit to him in New York This is confirmed by a letter from Koller to his daughter Mrs H Koller Becker quoted in her publication mentioned below (*38* 366) where he tells her that Nordenson (as chairman of the International Council of Ophthalmology 1933—1950) had arranged for Koller s preliminary

communication on cocaine to be published in an English translation in 1934 on the 50th anniversary of his great discovery and also that special attention was given to the jubilee at the meeting of German ophthalmologists in 1934 Besides the letter contains the following lines about Nordenson who mother says, is the best looking man she ever met which indicated that Nordenson had been at Koller s home

I got the opportunity to read the letters from Koller to Erik Nordenson found them very interesting and proposed that they should be published Wilhelm Nordenson gladly consented but since he felt very tired he asked me to attend to this matter At about this time Dr Holmstedt and I received a letter, dated August 21, 1964 from a lady unknown to us who lived in Highland Park Ill She was the above mentioned daughter of Carl Koller She told us that P Knoepfel Professor of Pharmacology at the University of Louisville, had called her attention to our book and what she was kind enough to characterize as its excellent and accurate account of the discovery of local anesthesia in surgery by my father Enclosed were two copies of a comprehensive article Carl Koller and Cocaine that she had written and published at about the same time that our book appeared *(38)* Her report which will often be quoted in the following pages turned out to be a very well written and carefully documented account To a great extent it is founded on papers left behind by her father among them several earlier unpublished letters from Sigmund Freud to him as well as letters from Koller to his daughter The first named letters had been translated into English with the cooperation of Dr Ernst Freud a son of the great psychoanalyst When reading the article one could hardly avoid finding that the case of Koller was to a certain extent analogous to several others which have bestowed on the Vienna medical faculty an unenviable notoriety e g those of Semmelweis and of Landsteiner In this situation it seemed desirable to get into print as soon as possible the letters to Erik Nordenson who seems to have been a kindred spirit to Koller with regard to independent judgment as well as fearlessness At the end of November 1965 the relevant number of the journal appeared *(46)* — the cover has the date of September but that was evidently what had been hoped for which as is well known does not always coincide with the result In the following we will find that particulars about the date of publication ought sometimes be taken with some caution

From Koller s letters one gets the impression of a highly intelligent and temperamental person with strong scientific interests and independent judgment But it also becomes evident that Koller at the beginning of this time (1885–1886) felt himself persecuted in Vienna and depressed The causes of this will be illustrated later in this paper He probably met Dr Erik Norden

son at the Versammlung deutscher Naturforscher and Ärzte (Assembly of German natural scientists and physicians) in Strassburg in September 1885 In a letter (38, 354) Freud had advised him to go there—partly because there may be a market in which someone would buy you Erik Nordenson who had been abroad for many years studying ophthalmology during the period 1883—1887 lived in Gottingen engaged in an extensive investigation of the detachment of the retina (ablatio retinae) at the clinic of Professor Th Leber Nordenson had attended the meeting at Heidelberg in September 1884 when Koller s discovery of the value of cocaine for anesthesia of the eye was reported and demonstrated It was no doubt Nordenson who arranged a sojourn for Koller at Leber and it was also certainly his personal acquaintance with Donders and Snellen in Utrecht which led to a more than a year long engagement there The zest for life now returned to Koller and Freud wrote (38 357)

> "With the greatest pleasure I see from your letter what a warm interest you take in me and I conclude further that a gratifying change has taken place in you since I saw you last at the peak of your illness which now that I am riper in experience

— Freud had just spent several highly appreciated months in Paris with Charcot —

> I can certainly diagnose as neurasthenia

Both Freud and Nordenson advised Koller to emigrate to the U S A and after some hesitation which was removed after a visit to Vienna he departed via London where he spent some days for New York arriving there May 18 1889 He spent the following 55 years in that city as a highly appreciated eye practitioner and consultant at several hospitals His old inclination to scientific work remained and in spite of the somewhat unfavourable circumstances he published a number of papers demonstrating his keen power of observation his energy and broad knowledge Thus he described several rare and even fatal eye diseases he demonstrated the nerves of the cornea and he also took up problems about astigmatism its determination and the form of the retinal images in this situation He contributed to the therapy of certain eye diseases e g by blood letting Furthermore he constructed an improved system of illumination for the electric ophthalmoscope (1918) A paper on cocaine in eye operations (35) will be considered later on

Like many others perhaps more in those days than now, during the beginning of his stay in New York Koller felt convinced that the Americans were greatly inferior to the Europeans with regard to education and civilization but he soon learnt to appreciate the American people their great

hospitality and readiness to help love of freedom and efficiency without over looking the shortcomings which must necessarily be incident to such a hetero genous nation

It now seemed that a thorough analysis of the role of Koller would be desir able from the standpoint of the pharmacologist and consequently when I was asked to write an introductory chapter about the history of local anesthesia for the volume on local anesthesia for the great International Encyclopedia of Pharmacology and Therapeutics I readily accepted this invitation *(47)* It is to some extent on the analysis made there that this report rests though many new details are given here

The introduction of general anesthesia or narcosis is rightly considered as one of the greatest advances made within medicine in the 19th century together with antisepsis and asepsis it forms the foundation for the splendid development of surgery in our time Here an exact dating can be given viz Thomas Green Morton s famous demonstration of the practical usefulness of ether narcosis for operative purposes taking place at the Massachusetts General Hospital at Boston on October 16 1846 The following year James Simpson Professor of Obstetrics at Edinburgh proposed the use of chloroform instead of ether and later on there have been continuous endeavours to find new means for general narcosis with lessened risks To day this speciality has advanced a great deal in many cases certain derivatives of barbituric acid are in the foreground owing to the quick and easy induction—they are given intravenously— and the short duration of their action It is characteristic for the situation however that in 1848 thus shortly after the introduction of chloroform Simpson himself pointed out (67) that if we were able to call forth local anesthesia without the unconsciousness that accompanies general narcosis many would greet this as still greater progress He also made some crude experiments to reach the desirable goal using the vapours of chloroform ether carbon bisulphide and even hydrocyanic acid Some years later the surgeon A Richet in Paris *(61)*, father of the Nobel laureate Ch Richet emphasized the danger of chloroform narcosis even in the hands of exper ienced physicians and tried with some success to use ether for local anes thesia but this time employing its cooling effect when vaporizing it

Just as general anesthetics have old ancestry in the form of opium Man dragora and palm wine all of them dangerous at a time when great un certainty prevailed regarding the dosage attempts have been made for cen turies to call forth local anesthesia sometimes not without a certain amount of success I will only give a few hints here

It is well known that hypnosis and several of its forerunners such as in cantations amulets the influence of sorcerers and medicine men might give a

blunting of pain sensation and at least during smaller operations be of considerable value For a long time it was also believed that continuous pressure on certain nerve trunks ought to be used as a means of producing anesthesia in the innervated area usually an extremity As an example the short description given by Ambroise Pare (1510—1590) the famous military surgeon of the 16th century may be mentioned He emphasized that powerful squeezing gives rise to three advantages viz better possibilities of covering the bone stump after an amputation the stopping of bleeding and a reduction of pain (*57*) It is characteristic of Pare s good judgment that he mentioned the reduction of pain but not its disappearance The explanation of the unsatisfactory results of the squeezing has been given in modern times by Gasser and Erlanger who received the Nobel Prize in Physiology or Medicine (1944) for their discoveries relating to the highly differentiated functions of single nerve fibres Using the cathode ray oscillograph which they had introduced into physiology they proved that the conduction velocity in the single fibres is approximately a linear function of the diameter of the fibres concerned Blocking the conduction with pressure was found to give effect at first on fibres with large diameter i e motor fibres whereas the fine fibres conducting pain impulses were affected last On the other hand these fine fibres were affected first by a cocaine solution presumably due to the relatively great surface area as well as by cooling (*22*) As already indicated cooling has been used in order to obtain local anesthesia Here another great military surgeon D J Larrey (1766—1842) Napoleon s head surgeon in La grande armee has published practical results of considerable importance (*42*) Thus he described that he had operated mainly performing amputations under very cold conditions (—19° C) when his assistants were hardly able to hold the instruments but dropped them to the ground He found it striking that the patients recovered with unexpected rapidity and immediately after the operations felt much relieved Larrey s description seems to be the first demonstration of the valuable effect of hypothermia during operations probably because shock is avoided and the central nervous system is relatively little affected

As we all know it is with the aid of chemicals that local anesthesia has gained its greatest victories The progress is naturally tied to the development of chemistry Alcohol was the first of the substances used for this purpose sometimes it is used even to-day e g for injection in the nerve trunk in unbearable neuralgia of the trigeminal nerve But in this case high concentrations are used (over 50 %) and the nerve is thus killed The starting point for modern local anesthesia is as is well known the alkaloid cocaine for the first time obtained in the pure state from the leaves of the Coca bush by A Niemann (*52*) in the laboratory of F Wohler in Gottingen Niemann also

morphine The consequence was the one so often met with—he became a morphine addict His friends tried to help him in different ways and when Freud had found reports in American literature about the successful use of cocaine in such cases in order to get rid of the addiction, he decided to try such treatment on his friend The result at first seemed excellent and Freud felt convinced that here was a great discovery that he would like to exploit and develop As time went on however the patient showed an increased need for the new remedy and soon chronic cocainism had succeeded the morphinism This seems to have been the case already before 1886 for Koller tells us in a lecture from 1940 (*38* 326) that 'many a night have I spent with him watching him dig imaginary insects out of his skin in his sensory hallucinations , thus typical symptoms of chronic cocainism had developed Freud who as mentioned collaborated with Koller though nothing of value resulted was now deeply interested in the wonderful drug cocaine and went through the literature the result being published in a summing up which from several points of view is excellent and came in print in July 1884 (*19*) Recently an English translation has become available (*19b*) He there refers in detail to the animal experiments already performed by Moreno and by von Anrep from which it appeared that at first a stimulating and later a paralysing effect was obtained Of course he also gave much attention to the general effects on man as described e g in the classical papers by Mantegazza and others as well as the findings of the old Christison in experiments on himself where a considerable improvement of the capacity for work became established with cocaine e g during mountaineering—in good agreement with the experiences with coca leaves of the natives in South America Freud mentioned after Niemann that cocaine produces an anesthetic effect on the mucous membranes (*19b* 5) With regard to his own observations on this point he wrote (*19b*, 9)

> A few minutes after taking cocaine one experiences a sudden exhilaration and feeling of lightness One feels a certain furriness on the lips and palate followed by a feling of warmth on the lips and cold in the throat On other occasions the predominant feeling is a rather pleasant coolness in the mouth and throat

The effects with regard to the disappearance of pain sensations after pin pricks is not mentioned It is also characteristic that Freud does not refer to or comment upon the observations on themselves by Moreno or von Anrep quoted above nor of their conclusions about the possibility that cocaine might be used to induce local anesthesia e g by direct injection

In the last chapter of his review Freud summarized the different forms in which cocaine might be used with advantage in therapeutics The following possibilities are mentioned *a*) as a stimulant *b*) for digestive disorders of the stomach *c*) in cachexia *d*) in the treatment of morphine and alcohol addic

The measurement of secretion and absorption by intestinal loops in conscious dogs

By L M VAUGHAN WILLIAMS *Department of Pharmacology, University of Oxford*

An operation for the introduction of cannulae into both ends of a Thiry Vella intestinal loop, and an apparatus for measuring the rate at which fluid is transported along it has already been described (Streeten & Vaughan Williams, 1951) The cannulae used were made of silver and it was not found possible to prevent the ultimate development of leaks around them Recently, with

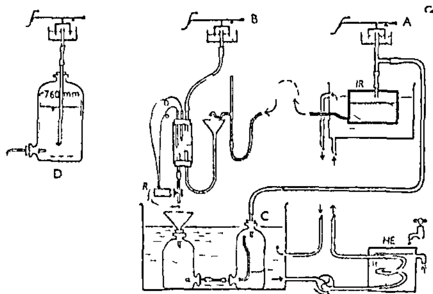


Fig 1

plastic cannulae of slightly modified design, such leakage has been eliminated In the absence of any loss of fluid between the loop and recording equipment, it became possible to extend the method to the measurement of rates of secretion or absorption by the loop

In principle the apparatus (Fig 1) is very simple One float recorder (A) measures the rate at which fluid enters the loop and another (B) the rate at which it leaves

Fluid flows from a reservoir whose height is adjustable, through a heat exchanger (not shown), which raises its temperature to 38° C, into the proximal end of the loop It emerges from the distal end, and, if expel[illegible] sufficient force runs out of a side arm in a vertical glass tube, w[illegible] is also adjustable The volume of fluid expelled is recorded by [illegible] (B) connected to a glass cylinder which, whe[illegible] is filled [illegible] level, is emptied by a relay (R)

The addition of another recorder to [illegible] rate [illegible]

tion *e*) as an aphrodisiac *f*) local application This last section, having played a special role in the discussion will be quoted in full

> Cocaine and its salts have a marked anesthetising effect when brought in contact with the skin and mucous membranes in concentrated solution this property suggests its occasional use as a local anesthetic especially in connection with affections of the mucous membranes According to Collin Ch Fauvel strongly recommended cocaine for treating diseases of the pharynx describing it as *le tenseur par excellence des chordes vocales* Indeed the anesthetic properties of cocaine should make it suitable for a good many further applications

Freud s description of the local actions of cocaine is both incorrect and incomplete thus demonstrating that his real interest in this aspect must have been rather insignificant in comparison to the general effects to which he obviously attached great weight Among the errors one first observes Freud s statement that cocaine has an anesthetic action on the skin which he does not substantiate in any way from the literature There is in fact no such effect It is of special interest in this connection that Wagner (later known as Wagner Jauregg) in the beginning of 1886 published a paper (*68*) describing a method of obtaining anesthesia of the intact skin with cocaine His starting point was a publication from the preceding year by Rusconi (*62*) who had written that painting the skin with a two per cent aqueous solution of cocaine hydrochloride within two minutes provoked an anesthesia so that needle pricks were hardly felt and a piece of ice placed on the skin was only felt after a minute by the subject The effect became still stronger with a three per cent solution in alcohol With this painted on the skin deep seated pain e g at parametritis could be abolished and it even permitted incisions and small operations without any pain One wonders whether Wagner took up the problem because he had observed the error made by Freud and preferred to discuss the question without mentioning the mistake of his friend Wagner stated that he had not succeeded in obtaining anesthesia of the intact skin with cocaine solutions even after ten minutes and also using much stronger solutions By the aid of cataphoresis of cocaine solutions in water—the method had been introduced by Gartner—it became possible for Wagner to obtain complete anesthesia of the skin so that an incision through it was not felt This communication by Wagner seems to have stimulated others and soon afterwards A Adamkiewicz (*1*) tried the method with chloroform and came to the conclusion that it was possible by cataphoresis to obtain skin anesthesia also with this substance H Paschkis and Wagner found this very improbable (*58*) since chloroform carries practically no electric current and they proved convincingly that the observations of Adamkiewicz had been erroneously interpreted (*58*) They warned directly against the local use of chloroform since it may give rise to

rather profound etching (as we to our discomfort have convinced ourselves But the anesthetic effect of cocaine is not the only mistake made by Freud the same is true with regard to his statement that cocaine and its salts have an anesthetic effect on mucous membranes since the effect in this case is practically restricted to the salts as discussed later (p 20) Furthermore Freud stated that to be efficient the solutions should be concentrated but as mentioned above von Anrep found good effect from 0 6—1 per cent solutions The results from subcutaneous injections by Moreno and by von Anrep seem to have been completely unknown to Freud though he quoted both papers In other connections Freud mentioned the subcutaneous applications of the substance for therapeutic purposes (*19 b 15*) but his curiosity seems to have been limited to the reduction of pain on other mucous membranes than those already tried in this direction As a basis for the assertion that Freud has given Koller the impulse to his decisive experiments on the surgical application of cocaine anesthesia one must conclude that it is very meagre much more so than shown by the earlier literature

Having finished his review Freud made a journey but before leaving Vienna he spoke to his friend Dr L Konigstein assistant professor in ophthalmology asking him to take up the question of the practical use of cocaine in connection with diseases of the eye Koller who had had practical personal experience of the numbing effect of cocaine hydrochloride on the tongue and the mucous membrane of the mouth during his experiments with Freud asked himself whether this might be the substance he had been looking for without success He went to Stricker s laboratory and tried it first on a frog then a guinea pig a rabbit and a dog and when all these experiments clearly established its efficiency in producing local anesthesia corresponding trials were performed with the same success on himself and the assistant Dr Gartner (*21* quoted from *38* 331) who warmly congratulated him to the important finding

Being naturally anxious to have his results known in scientific circles and the priority protected Koller wrote a brief summary and asked his friend Dr A Brettauer from Trieste to read it at the meeting of the German ophthalmologists in Heidelberg on September 15 and 16 1884—economic difficulties made it impossible for Koller to go himself

In this classical report now available also in English translation (*33*) Koller carefully described the anesthesia of the conjunctiva and the cornea after instillation of a few drops of a two per cent aqeous solution of cocaine hydrochloride into the conjunctival sac its rapid onset with the abolition of all reflexes from the cornea as well as its relatively slow disappearance In animals he proved that the anesthetized cornea could be scratched or scraped even perforated or cauterized with silver nitrate until it became completely white

and also be cut without any reaction from the animal Also after the production of a foreign body keratitis the effect of cocaine was apparent In man the local anesthesia from cocaine was accompanied by a widening of the palpebral fissure as well as for the pupil though the dilation of the pupil was not maximal A slight paresis of the accommodation was also observed

In conclusion Koller wrote

> Perhaps it would not be presumptuous if I expressed the hope that cocaine can be used successfuly as an anesthetic agent in removal of foreign bodies from the cornea in more extensive operations or as a narcotic in diseases of the cornea or conjunctiva Since the experiments I have just described were carried out only during the past two weeks *I must reserve for later publication a consideration of other papers which possibly concern this question* and an evaluation of their contents Vienna beginning of September 1884

This report a model of exact and concise description careful conclusions and hopeful optimism was very well received at the Heidelberg meeting especially after Dr Brettauer had demonstrated the remarkable effect of a solution of cocaine hydrochloride on the eye of one of the patients from the Eye Clinic The only person who expressed himself in the discussion was Dr H Schmidt Rimpler professor of ophthalmology at the University of Halle who said The new substance will *if it is borne out further be of the utmost* importance for our operations Dr H D Noyes professor of ophthalmology in New York who had been present at the Heidelberg meeting immediately wrote a letter to an American colleague who tried cocaine on October 8 though the result was not made known before much later (*50*) But Noyes also published a report from the meeting in a well known journal (*55*) where he wrote

> Perhaps the most notable thing was the exhibition to the Congress on one of the patients of the Heidelberg Eye Clinic of the extraordinary anesthetic power which a two per cent solution of muriate of cocaine had upon the cornea and conjunctiva when dropped into the eye The momentous value of the discovery seems likely to prove to be in the eye practice of more significance than has been the discovery of anesthesia by chloroform or ether in general surgery or medicine

The later publication indicated by Koller in the words quoted above took the form of a lecture read before the Wiener Medizinische Gesellschaft (Viennese Medical Society) on October 17 1884 and published soon afterwards (*34*) He could then state that his results had already been confirmed in various places in Germany He now referred to some older observations of the numbing effect of cocaine hydrochloride on the tongue and the palate performed on himself by C D Schroff in 1862 (*64*) he further mentioned von Anrep s comprehensive account of experiments with cocaine and also

in contrast to Freud—that he had pointed out at the end of his paper that the anesthetic action of cocaine might be of importance

> For us Viennese he continued cocaine has become a favorite topic of discussion because of the thorough review and interesting work of my colleague at the Allgemeines Krankenhaus (General Hospital) Dr Sigmund Freud

Koller was also able to make some important additions to his first report such as ischemia of the conjunctiva after cocaine as well as widening of the palpebral fissure, occurring before the action on the muscles in the iris and the ciliary body and therefore attributed by Koller to removal of stimuli He also concluded from the late effects on the pupil and the accommodation that a slow absorption of the cocaine solution had taken place Still more important was the fact that Koller could refer to clinical observations—thanks to the kindness of Professor A L von Reuss who was the temporary chief of the second Eye Clinic at the hospital—which clearly demonstrated the practical value of cocaine solutions in the treatment of phlyctenular conjunctivitis with eruptions and ulcers of the cornea, as well as the prevention for a few hours of pain from cauterization of the lids with silver nitrate He also had used cocaine successfully in several operations such as removal of foreign bodies from the cornea further in iridectomy and even an extraction of cataract without the patient feeling any pain

Koller s lecture was followed at the same meeting by a communication from Konigstein (see below) Professor Lesky in her valuable book (*44* 489) Die Wiener medizinische Schule im 19 Jahrhundert says that Konigstein s contribution was delivered on October 19 thus 2 days after Koller s but there is no doubt that it occurred at the same date as Koller s lecture as seen from the minutes of the meeting (*40*) and also from Koller s own words (*38* 341) October 19 is the date of the publication of the first half of Konigstein s lecture (*39*) At the same meeting (Oct 17) Dr Hock and Professor Reuss confirmed the absolutely striking success with cocaine The latter advised all ophthalmologists to make no operations before the application of cocaine This must be applied directly on the cornea and for long time It can be used also by glaucoma

A week later (the minutes *40* 1315 have been wrongly dated to Oct 25 instead of Oct 24 cf Jelinek *30*) Jelinek spoke in the society about painless operations in the laryngeal region under the influence of cocaine which he had tried at the advice of Koller In the discussion Professor L von Schrotter pointed out the value of the new method for laryngoscopy A little later E Frankel (*18*) demonstrated its usefulness on the genital mucosa But this was only the beginning Before the end of the year 1884 numerous letters to the

editor had appeared on the successful use of cocaine as a local anesthetic in the Lancet and the lively interest was clearly demonstrated from many countries Perhaps this is best illustrated from U S A The New York ophthalmologist H Knapp editor of Archives of Ophthalmology, at the end of the year published an article there starting with the following words

> No modern remedy has been received with such general enthusiasm none has been so rapidly popular and scarcely any one has shown so extensive a field of useful application as cocaine the local anesthetic introduced by Dr C Koller of Vienna

Knapp then gave a translation of Koller s lecture before the Viennese Medical Society

> not only as an acknowledgment of a debt of gratitude we all owe to him but also as an appropriate introduction

Then followed the discussion of about 60 communications from American physicians who had employed cocaine anesthesia successfully He also told about corresponding results from Germany France and Great Britain In conclusion he said that cocaine had been found useful in Ophthalmology Otology Rhinology Laryngology, Pharyngology Urology Gynecology and also in general Surgery (*32*)

The slow absorption of the cocaine solution into the interior of the eye from the conjunctival sac is a drawback and necessitated during the early days of local anesthesia preparation of the patient long before the operation In a paper published in U S A in 1892 (*35*) Koller wrote

> Very soon after my first communication I began using sub conjunctival injection in squint operations and during my time of assistantship to Professor Snellen performed many tenotomies and advancement of muscles all with conjunctival applications of cocaine (after the conjunctiva had been anesthetized in the usual way) The operation can be made perfectly painle s and we have the double advantage of sparing th pain to the patient and not bringing discredit on a good drug

It was of course to be expected especially after von Anrep s experiments on himself that the subcutaneous application of the drug would also soon be tried The question was taken up by the young American surgeon W S Halsted (1852—1922) later called one of the leading lights of surgery in this country (*49*) it has also been said about him that probably no one man has had a more profound effect upon American surgery (*29*) Among his great contributions are also several concerning the use of cocaine in general surgery For this he had to pay dearly becoming innocently an addict to cocaine at a time when the risks of using it were unknown and had great difficulties in getting free from his illness His contributions are not well

enough known everywhere In a letter to the editor of the New York Medical Journal R J Hall (*25*) described some cases in which he assisted Halsted where cocaine was sucessfully used subcutaneously or even to block certain nerves thus calling forth regional anesthesia In a letter from Halsted to W Osler (*26*) written many years later (1918) Halsted reports about his early successful use of cocaine anesthesia in major operations such as amputations excision of joints etc, as well as the blocking of nerves and the effect of anemia in prolonging the anesthetic action He also mentioned that he had demonstrated the application of cocaine in general surgery to Dr A Wolfler Billroth s first assistant during a visit to Vienna in the autumn of 1885 According to Halsted, Wolfler had declared cocaine worthless in surgery, but after Halsted had shown him he became enthusiastic on the subject In another letter of 1920 (*27* 172—174) to C L Kells some further details are given Halsted states there that he started to try cocaine—in collaboration with numerous students—one or two weeks at most, after the arrival of Koller's report The valuable results of blocking the nerves for dental surgery were confirmed by E H Raymond (*59*) The influence of Halsted on Wolfler is mentioned by the latter (*70*) in the following terms

> In the beginning of November this year I had a visit from the practising surgeon in New York Dr Halsted who told me he had injected cocaine along the nerves concerned subcutaneously and thus obtained anesthesia Therefore about a month ago I took up again the cocaine injections though I did not care further about the course of the subcutaneous nerves

He then mentioned that shortly before this A Landerer (*41*) in Leipzig had published his experience of the successful use of cocaine injections subcutaneously in patients Thus when E Lesky states (*44* 489) that it was Wolfler who made cocaine fruitful for surgery this cannot be upheld neither can her conclusion that cocaine anesthesia was the original work of the medical youth of Vienna if not Koller *alone* is taken as the representative of the youth Corning (*11* *12*) and Bier (*4*) share the merit of having shown the applicability of anesthesia by injection of local anesthetics into the cerebrospinal canal and several other methods of application have also been introduced

There exists a remarkable parallelism between the situations after the introduction of general narcosis and of local anesthesia This even involves certain personal experiences of the men who were primarily responsible for the great progress In both cases it is true that their achievements did not contain a new revolutionary idea since the fundamental observations had been done long before Quoting once again the lecture by Clark (*10*) one must admit that

there i however a world of difference between a bright idea and a suc ces ful achievement and the main credit must always go to the man who pos sesses the energy determination and skill to demonstrate the possibility of progress along some new path

At the same time however controversial priority claims are more easily stated in such situations Thus Professor M J Rossbach of the Pharmacological Institute of Wurzburg where von Anrep carried out his work on cocaine protested that this had been ignored by Koller In this however Rossbach was mistaken the error being due to the fact that he had not read the original of Koller s lecture but only a review of it (*38* 342—343)

As already mentioned (p 16) Konigstein spoke immediately after Koller before the Viennese medical Society on October 17 1884 giving a complete communication of his own experiments on the anesthetic effect of cocaine on the eye He mentioned that on himself and other subjects he had observed that the conjunctiva and the cornea became anesthetised after the application of a one per cent cocaine solution that the pupil dilated moderately after about ten minutes and that accommodation decreased somewhat He had tried cocaine with excellent results in extraction of foreign bodies from the cornea in incisions of chalazions etc Pains from phlyctenular and pustular processes in the eye as well as vesicles fom herpes zoster in the eye had also been treated with good results During the days immediately before the meeting he had extracted an eye from a dog under cocaine without any sign of pain Konigstein however did not mention Koller s priority although it had been known for more than a month that he had made similar observa tions and had had them demonstrated in Heidelberg Freud and Wagner Jauregg friends of both Koller and Konigstein succeded in persuading him to withdraw his claims on priority When he published his own lecture in the Wien med Presse in the issues of October 19 and 26 (*39*) he did not say anything about his omission but simply added the following sentences at the end of the paper

"It remains to define my relations to the communication by Dr Koller on Sep tember 15 at the Congress in Heidelberg and with regard to the fact that the complete anesthesia of the cornea and conjunctiva from cocaine were then for the first tim pronounced I started my investigations independently of Mr Koller but I ad at the time of his communication not arrived at a definite judgment regarding the anesthesia from coca also observed by myself My further experiments soon gave me confirmation of Koller s statements though other observations ment oned here were absolutely independent

One can well understand that Jones in his well known biography on Freud (*31* 95) noted that Konigstein made his statement reluctantly Jones asser tion is in fact an understatement and ought to be confronted with Koller s

statement (38 341) that Konigstein when he heard that Koller had declared cocaine a perfect anesthetic for the eye operations said that he was mistaken In this connection it seems of special interest that Konigstein in his article (39) mentions that in one case he had obtained a cocaine solution which was without any effect In this case the pharmacist had added too much acid in order to quicken the dissolving of the substance, and Königstein supposed that the irritating effect of the acid counteracted the anesthetising one of the cocaine To-day it is of course well known that the real explanation is different It is the free base cocaine that is efficient owing to its lipid solubility in contrast to the salts which are necessary for the absorption But if the reaction is acid the dissociation becomes reduced and the effect will more or less disappear It can under these circumstances not be excluded that Konigstein after his negative result was more or less directly stimulated by Koller s success

Jones has mentioned the intervention by Freud and Wagner Jauregg concluding in the following terms As we shall see Koller did not reciprocate Freud s chivalrous behaviour (31 95) It is a little difficult to find it chivalrous by Freud and Wagner Jauregg that they reacted against Konigstein s attitude Freud had asked him to try cocaine to alleviate the pain of certain eye complaints, such as trachoma and iritis' (31, 95) but without any idea that it could be successfully used at operations and his review on this point was as we have seen especially inadequate He became as stated in a letter from him to Koller (38 342)

> aghast at the fact that in K s published paper there is no mention of your name and I don t know how to explain it in view of my knowledge of him in other respects but I hope you will postpone taking any steps until I have talked to him and that you will after that create a situation in which he can retract

To this might be added that Wagner Jauregg who knew Stricker s laboratory well also intervened He was already known in those days as upright fair minded and fearless

After reading the sentence quoted from Jones it is with some interest that one goes to his motivation and I quote his criticism in full (31 96) After mentioning that Koller emigrated to U S A and had

> a successful career (?) there

he said

> But even at the beginning of his achievement he committed a symptomatic error which indicated some disturbance in his personality that came to open expression in later years When publishing the paper he had read in Vienna in October 1884 he quoted Freud s monograph as dating from August instead of

July giving thus the impression that his work was simultaneous with Freud s and not after it Both Freud and Obersteiner noticed this slip and corrected it in subsequent publications As time went on Koller presented the discrepancy in still grosser terms even asserting that Freud s monograph appeared a whole year after his own discovery which was therefore made quite independently of anything Freud had ever done

Perhaps we may correlate this curious behaviour with the fact that in hospital days Freud had treated him privately for a neurotic affection negative trans ferences as they are called often endure

It is certainly rare to meet in scientific literature such grave accusations with so little factual foundation In the following all points concerned will be taken up and their real value will be illustrated Jones starting point is that in quoting Freud s monograph (incomplete review would have been more exact) as dating from August instead of July Koller gives a certain impres sion But in his Vienna lecture he explicitly emphasized that the review and the therapeutic results of Freud had called the attention of the Viennese doctors to cocaine This is also quoted by Jones (*31* 95) How can it then be possible that Koller s work should have been simultaneous with that of Freud? Every careful reader will understand that Koller had not the least desire to deny that Freud s work had an *indirect* influence on his own Both Freud and Obersteiner noticed the slip and corrected it in subsequent publi cations Jones adds In foot note 1) to his paper of January 31 1885 (*20*) Freud mentions the date of his publication of the review adding in brackets (not August as often incorrectly cited) without however especially calling attention to Koller or to anybody else Obersteiner (*56*) on the other hand gives the correct dating but without in any way complaining of errors from others—it is a pure guess that he tried to correct the slip An illustration that Koller is not alone with regard to the slip is given by Konigstein who naturally acknowledged that he had been directly inspired by Freud and made exactly the same mistake as Koller without however being accused of a symptomatic error It seems probable that a common factor is responsible for the error e g that the reprints of the review became available or were sent to the friends of Freud rather late The original article contained neither date of reception nor of publication Furthermore Koller in his preliminary report to Heidelberg stated that he had performed his experiments the last weeks of August or even the beginning of September Without a shred of evidence Jones a little earlier (*31* 94) tells us that Koller read Freud s essay when it appeared in July When continuing his discussion of what he calls the dis crepancy of Koller Jones gives as reference Koller s paper of 1935 (*37*) From this brief communication which is an addition to the paper published

interest

> At this time I was asked by Freud to undertake together with him a series of experiments in order to test the effect of cocaine on muscular strength and fatigue. These experiments gave no particular results.

Later in the article he points out that

> Moreno and ... von Anrep had both pointed out that cocaine might be of practical ...

The paper, however, did not contain one word of what Jones has charged Koller with. Thus the remarkable situation has arisen that the man who founded a grave charge against another person on a simple mistake in the publication ... (Aug. instead of July) himself when referring to another paper gives neither the publication nor even the journal correctly. I have been able to find the paper obviously meant by Jones. It is a letter from the 83 year old Koller to Dr. M. G. Seelig (66) which the latter published with Koller's permission. In this letter Koller mentioned that he had greatly appreciated an article by Seelig about the progress in medicine during the last hundred years. He then wrote

> Coming to the dealing with anesthesia I could not help being pained by your adopting the incorrect statements not of Freud himself but of his admirers and hangers on that Freud had anything to do with starting cocaine as a local anesthetic in surgery.

There followed a concise and correct report on the main steps of the historical development. Further on he wrote

> Freud in his Autobiography regrets the fact that he passed by this great opportunity and gives full credit to me. I find fault with him only in that he failed to call off his over enthusiastic admirers for giving credit to him for what he did not have any right to.
>
> The facts are that Freud did not have anything whatever to do with cocaine anesthesia nor did he write a single word about work on cocaine in 1885 (whereas my work dates from 1884) that had not been done better and more scientifically by Anrep in 1879. Historical untruths are very difficult to destroy.

In a P. S. Koller mentions his article of 1935 (37) which

> I published purposely in a Viennese medical weekly so that it would surely come under Freud's eye. I thought it might make him come and disavow the claims of his admirers that he had instigated my work on cocaine as a local anesthetic. However it had no such effect. I was on friendly terms with Freud and did not wish to accuse him directly of dishonesty. However his expression in his Autobiography that he had told me also of cocaine was so ambiguous as to cause all this untrue representation.

As these quotations clearly show it is not at all a question of Freud s review or monograph —about its indirect importance for stimulating people s interest in cocaine Koller had spoken several times before—but of Freud s own experimental studies published in some papers in 1885 the first and most interesting on January 31 1885 (*20*) i e several months after Koller s revolutionary paper Jones himself has pointed out the rather poor quality of this contribution by Freud at the same time making the most of it (*31* 101) By substituting the year 1884 for 1885 and by introducing the word monograph Jones has tried to substantiate his remarkable interpretation It is difficult to say whether Jones mode of procedure should be characterized as the outcome of want of understanding or of carelessness but it is obvious that it ought not to belong in a biography with scientific claims Jones finishes his criticism with a discreet suggestion that Koller s behaviour might be the consequence of an affection for which Freud had treated him in earlier days As een before (p 7) Freud in a letter to Koller, had pointed out that he had been suffering from neurasthenia in contrast to what Freud had said before Probably the majority of us would like Koller become neurasthenic if we were in danger of being deprived of the glory evoked by a great discovery and at the same time having the feeling of personal persecution In his book Jones also mentions that Knapp the well known ophthalmologist when meeting Freud greeted him as the man who had introduced cocaine to the world and congratulated him on the achievement (*31* 103) This remark highly exaggerated as it is does of course not imply that Freud had given the inspiration to the use of cocaine as an anesthetic in surgery On this point Knapp had as quoted above expressed in print the highest praise of Koller s work This may have been unknown to Jones In conclusion one finds that the criticism by Jones does not cast any shadow on Koller Unfortunately the same cannot be said about Jones and the author has sometimes wondered whether the art of psycho analysis could not with profit be applied to find an explanation for the numerous mistakes he has made

There can be no doubt whatever about the priority of Koller with regard to the introduction of cocaine in surgery Furthermore Freud himself in his paper (*20*) of January 31 1885 at a time when the whole story was fresh has written

> While Dr L Konigstein at my suggestion undertook to test the action of cocaine in alleviating pain and restricting secretions in pathological eye conditions Dr Carl Koller quite independently happened upon the felic tou idea of inducing complete anesthes a of the cornea and conjunctiva by means of coca n whose power to numb mucous membrane had been long kno vn

In spite of all the facts however the conception of a direct influence from

Freud on the initiative of Koller has still often been maintained e g in the monograph by Lofgren about xylocaine or lidocaine (*48*) 1948 in Bonica's great handbook (*6*) from 1953 and even in the recent work by Faulconer and Keys (*17*) in 1965 in spite of the fact that they quote in full Koller s letter to Seelig (p 771) A great responsibility in this connection rests on Jones whose erroneous presentation appeared many years after the death of Koller and consequently could not be answered by him It is indeed high time that the real situation was made clear

As mentioned before Koller has emphasized that Freud s work on cocaine focussed attention to the substance Lebensohn (43) has characterized this *indirect* influence in the following words after having mentioned the unsuccessful results of Freud s experiments in order to cure morphine addiction with the aid of cocaine

> Curiously just as ether anesthesia was derived from the bizarre notions of Beddoes on pneumatic therapy so local anesthesia was derived from this misconception of Freud

It is not astonishing that Freud personally regretted that he himself had missed the opportunity of making the great discovery that fell on Koller though it is probably true as pointed out by Jones (*31* 91) that it is not altogether likely that Freud even with more time at his disposal, would have thought of the surgical application one foreign to his interests In fact scientific creative work usually demands concentration about a more or less circumscribed problem with intentional elimination of disturbing complications As a recompense one will often find consolation in the conviction of having found something that others have passed over Freud thought that he himself was dealing with a great discovery in the treatment of morphine addiction

The parallelism between the two great discoveries of general narcosis and local anesthesia extends also to the unusually rapid acceptance of the new possibilities as well as to the insight that the methods are linked with certain dangers and have made new efforts necessary to build further on the fundamental discoveries To this it must be added that the men who were mainly responsible for the introduction of the new methods into practical medicine met with unpleasant personal hardships As is well known among the pioneers of general narcosis Morton died impoverished and embittered at the failure to obtain the reward that was discussed by the Congress of the U S A during 16 years whereas Wells committed suicide and Jackson ended his days in a mental asylum Only Long passed his life in relative tranquillity but he did nothing to make the discovery known to humanity as a whole but kept it to

himself The fate of Koller was also greatly influenced by his discovery In his letters to Nordenson (*46*) he complains of continuous depressing hostilities in Vienna obviously to a certain degree due to the prevailing anti emitism there In the beginning of January 1885 this found expression at an episode at the General Hospital Koller was in charge of the Admitting Room when a man with a seriously injured finger was brought in he saw that there was immediate danger of gangrene owing to the application of a tight rubber bandage around the finger A student from Billroth s surgical clinic asked that the patient be designed to this Koller made a note of his wish and then cut the bandage whereupon the other doctor called him Impudent Jew or according to Freud in a letter to his fiancée (*38* 318) Jewish Swine Freud continued

> Now you must try to imagine the kind of atmosphere we live in here the general bitterness — in short w would all have r acted just as Koller did by hitting the man in the face The man ru hed off der ounced Koller to the director who however called him down and categor cally took Koller s side — — — But since they both are reserve officers he i obliged to challenge Koller to a duel and at this very moment they are fighting with sabres under rather severe conditions

At the end of the letter Freud added

> Our friend is quite unharmed and h s oppor ent got two deep gashes We are all delighted a proud day for u

For Koller however the result increased his difficulties since he must leave the country duels being forbidden and thus also his hopes of obtaining the position of assistant at the Eye Clinic to which he otherwise would be very well qualified and an academic career for which he probably had hoped also became excluded It was during this period that Koller became depress ed It was also then that he met Nordenson and obtained his help in the continu ation of his training within ophthalmology and at last emigrated to New York During his long stay there he received many distinctions, such as several gold medals honorary memberships inter alia in Wiener medizini che Gesellschaft (Viennese Medical Society) 1930 He was several times proposed for the Nobel Prize in Physiology or Medicine His di covery was undoubtedly prize worthy but it was too old and had been generally accepted the same year and thus could not be considered at a time when the med cal faculty tried carefully to follow the statutes in awarding the prize to old r discoveries only in case their importance had been acknowledged recently

It is not possible here to describe the continued development of l anesthesia The general toxicity of cocaine has caused numerous deaths attempts have been made to avoid them by usin very dilute solutions

was emphasized at an early stage by Halsted (27 178) but the practical application became mainly the work of the Frenchman P Reclus (60) and the German C L Schleich (63) Halsted has pointed out (27 519) that after incising the anesthetized skin the underlying parts

> were comparatively insensitive to handling and for the most part even to cutting The accidental cutting or crushing of nerves caused the most exquisite pain and we noticed that the nerve supply of the blood vessels is so abundant that the severing or clasping of even very small bleeding points usually started a cry of some sort of remonstrance from the patient and now after many years of experience with cocaine we interpret an unexpected moan a signifying an insult to some small unseen blood vessel or nerve

Among the numerous new preparations introduced in the place of cocaine procaine (novocaine) has been especially valuable It was introduced by the chemist Einhorn (14) and the surgeon H Braun (7—9) The latter performed a comparison with other substances indicated a simple method for the assay and introduced the addition of epinephrine to such substances as —like procaine but in contrast to cocaine—cause dilation of vessels Together with Halsted Braun is certainly the clinician who has made the greatest contributions to the field Lastly it should be mentioned that the preparation which is at present most used for local anesthesia namely the Swedish discovered xylocaine (lidocaine) originated from the observation of the numbing effect on the tongue by a substance (15) by H Erdtman which gave rise to a large number of derivatives before the new remedy took form Thus the effect on the tongue this time was not overlooked but like Koller one understood to exploit the observation

On the ground once laid by Carl Koller an important branch of medical science and practical work has thus been successfully developed It is the old story of a young scientist who has been fascinated by an idea and thanks to this and his special training has been ready to take the opportunity when it has suddenly presented itself But it is also an illustration of the famous pronouncement by Pasteur that chance only favors those whose minds have been prepared At the same time Koller had the invaluable qualities of being true reliable and fearless (5)

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SUPPLEMENTUM 300

›IES ON THE BIOSYNTHETIC
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IN RAT LIVER

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ACTA PHYSIOLOGICA SCANDINAVICA
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FROM THE DEPARTMENT OF MEDICAL CHEMISTRY
UNIVERSITY OF HELSINKI FINLAND

STUDIES ON THE BIOSYNTHETIC PATHWAY OF POLYAMINES IN RAT LIVER

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HELSINKI 1967

To my wife

Preface

This work was carried out in the Department of Medical Chemistry University of Helsinki I am greatly indebted to my honoured chief Professor JOHAN JÄRNEFELT M D for his interest in my work and for placing all the facilities of his department at my disposal

I wish to express my deepest gratitude to my teacher and friend Dr AARNE RAINA M D for his continued support in the course of each stage of this study His interest and expert advice have been of inestim able help during my work

Further I wish to express my gratitude to Docent NILS ERIK SARIS Ph D and Docent ELJA PITKÄNEN M D for their criticism of the manuscript

I am grateful to my colleagues in the Department of Medical Chemistry In particular my thanks are due to my friend Dr MARTTI SIIMES for his intellectual and technical assistance during this work to Docent NIELS RÄIHÄ M D for many stimulating discussions and to Mr TIMO TELA RANTA B M and Mr OLLI JÄNNE B M for their advice at various stages of my work

I am very grateful to Mrs SIRKKA KANERVA for the skilful labora tory assistance which made my work possible

Miss ANN MATHESON M A has corrected the English text I thank her for her collaboration and interest in my work

Helsinki May 1967

JUHANI JÄNNE

CONTENTS

Introduction

Putrescine spermidine and spermine are aliphatic amines occurring in almost all living cells The wide distribution of these compounds has been known for years but the significance of their presence has not been established

Studies concerning the metabolism and effects of diamines and polyamines on cellular level have for the most part been carried out on microorganisms and the knowledge we have of their association with the intermediary metabolism of the mammalian tissues is rather meagre

Putrescine spermidine and spermine have unquestionably many effects on various cellular and subcellular components *in vitro* and most of these effects can be explained on the basis of their polycationic nature *i e* the stabilizing effects they exert on nucleic acids are in most cases comparable with those observed with higher concentrations of Mg^{++} or Ca^{++}

In recent years increasing evidence has been accumulated indicating that these compounds might also play an active role in many fundamental biological phenomena in mammalian tissues The changes taking place in the polyamine pattern during the growth processes of an animal tissue or organ whether developmental or regenerative makes it justifiable to assume that they are in some way related to cell multiplication and tissue growth

Review of the literature

Structure The diamine putrescine $H_2N(CH_2)_4NH_2$ is the decarboxylated derivative of the amino acid ornithine Spermidine $H_2N(CH_2)_4NH(CH_2)_3NH$ also contains in its molecule in addition to the putrescine fragment a propylamine moiety Spermine $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$ contains one putrescine fragment and two propylamine moieties

OCCURRENCE

Microorganisms Most of the studies dealing with the metabolism of putrescine spermidine and spermine have been carried out on microorganisms *Escherichia coli* a bacterium which has been studied in the greatest detail contains when grown in minimal medium 15 µmoles of putrescine and 1 5 µmoles of spermidine per g wet weight (TABOR and TABOR 1964) Part of the putrescine and spermidine in this bacterium occurs in the form of acetylated derivatives (DUBIN and ROSENTHAL 1960) The concentrations of these amines however vary markedly with the cultural conditions i e with pH (TABOR ROSENTHAL and TABOR 1958) Comparable concentrations of putrescine and spermidine have been obtained from various other bacteria (TABOR and TABOR 1964) There are however wide variations in the amounts found in different microorganisms In general it can be concluded that only gram negative bacteria contain putrescine and spermidine in the above-mentioned concentrations Most gram positive bacteria neither store nor excrete putrescine (HERBST WEAVER and KEISTER 1958) In contrast to the considerably high concentrations of putrescine and spermidine most microorganisms contain little or no spermine (TABOR and TABOR 1964)

In addition to the bacterium itself the bacterial viruses also contain putrescine and spermidine in high concentrations The neutralization of one third to one half of the deoxyribonucleic acid phosphate in the bacteriophage can be accounted by putrescine and spermidine The content of these amines however varies considerably with the type of the virus and in the first place with the putrescine and spermidine content of the host bacterium Further putrescine and spermidine can be replaced by other cationic compounds suggesting that these amines may act as unspecific cations neutralizing negatively charged phosphate groups of the phage deoxyribonucleic acid (AMES DUBIN and ROSENTHAL 1958 AMES and DUBIN 1960)

Putrescine and spermidine in addition to bacteria and viruses are naturally components in many plants and plant alkaloids The distribution of diamines and polyamines in the vegetable world is reviewed in detail by TABOR and TABOR (1964)

Animal tissues The first report of the occurrence of polyamines in animal tissues can be found in Lewenhoeck's letter to the Royal Society (see ref TABOR and TABOR 1964) The crystals he saw in human semen were probably spermine phosphate

One of the first systematic studies on the occurrence of spermine in various human tissues was reported by HÄMÄLÄINEN (1947) He observed that almost all human tissues contained spermine The highest concentrations of this polyamine were found in prostate pancreas and bone marrow The results of this study were generally in agreement with those earlier reported by DUDLEY and ROSENHEIM (1925) WREDE (1926) and HARRISON (1933)

The lack of adequate analytical methods at that stage prevented the study of small tissue samples and small laboratory animals The introduction of a sensitive method by ROSENTHAL and TABOR (1956) made it possible to determine the polyamine content in various tissues and organs of rats mice guinea pigs and rabbits This study included only a few tissues and organs but some general conclusions could be drawn First in most of the tissues studied the molar concentration of spermidine exceeded that of spermine Secondly there were great variations in the polyamine content in different tissues of the same animal e g rat pancreas contained 8 6 μmoles of spermidine and 1 0 μmole of spermine per g wet weight rat testis however only 0 4 and 0 5 μmoles respectively Rat liver and in particular rat prostate contained polyamines in high concentrations The amounts of polyamines in some experimental tumours varied but generally a high molar ratio of spermidine to spermine could be observed

The polyamines spermidine and spermine are also found in animal blood (ROSENTHAL and TABOR 1956 RAINA 1962a SHIMIZU KAKIMOTO and SANO 1965a) As stated by ROSENTHAL and TABOR (1956) spermine in the blood occurs in the formed elements and none is found in the plasma This fact which also holds true for spermidine was later confirmed by RAINA (1962a) using human blood SHIMIZU KAKIMOTO and SANO (1965a) assumed that spermine occurs in or around the cell nucleus This idea was based on the findings indicating that the concentration of spermine was considerably high in the blood of non mammals whose erythrocytes have nuclei

The association of diamines and polyamines with growth processes is an interesting feature in the metabolism of these compounds in animal tissues None of diamines or polyamines could be detected in unincubated eggs (RAINA 1963 CALDARERA BARBIROLI and MORUZZI 1965) On the other hand the concentrations of spermidine and spermine in the embryos increased sharply during incubation reaching two typical peaks during the 2nd — 3rd and 15th — 16th days of incubation (RAINA 1963) In addition to the polyamines the diamines putrescine and cadaverine (decarboxylated derivative of lysine) also showed similar changes during the development of the chick embryo (CALDARERA BARBIROLI and MORUZZI 1965) These changes in the polyamine pattern correlated very well with those taking place in other areas of embryonic metabolism e g the concentrations of nucleic acids showed parallel behaviour at each stage of the development of the chick embryo (CALDARERA BARBIROLI and MORUZZI 1965)

Based on the above investigations it seems that the age of an embryo or animal is an important factor affecting the metabolism of diamines and polyamines This fact was later confirmed by determining the concentrations of polyamines in various rat tissues in relation to age (JÄNNE KAINA and SIIMES 1961) Again great variations between different tissues of the same rat was observed but some generalization could be made Metabolically active and cell rich tissues such as the liver and thymus of a young animal contained much more spermidine than spermine Further in all the tissues studied the concentration of spermidine decreased without exception with increasing age The decrease was in general most marked during the first month of life The changes in the concentration of spermine were considerably smaller but an increase in the concentration of this polyamine was observed mostly during the first month The changes in polyamine content with increasing age were reflected by the molar ratio of spermidine to spermine In the liver for instance this ratio was 4 52 at birth then decreased steadily being only 0 84 9 months after birth This decrease in spermidine and usually a slight increase in spermine was a uniform feature in all the tissues studied Similar results were later reported by SHIMIZU KAKIMOTO and SANO (1965b) using some mice tissues A correlation between spermidine and deoxyribonucleic acid was also observed

In contrast to the investigations dealing with the occurrence of spermidine and spermine in animal tissues there are only a few studies concerning the distribution of the diamine putrescine in the animal world This diamine is in fact the main aliphatic amine in bacteria and bacterial viruses and as will be seen later acts as a quite effective precursor of spermidine biosynthesis in animal tissues too There are reports of the isolation of diamines from animal material but these were in most cases cadavers undergoing bacterial decomposition (GUGGENHEIM 1951) The concentration of putrescine found in fresh tissues of some mammalian species has been reported to be very low as compared with the concentrations of spermidine and spermine According to WEAVER and HERBST (1958) the concentration of putrescine in liver of several species was only about 1/50 of the spermidine and spermine concentration The concentration of putrescine compared with that of polyamines was still lower in the porcine and bovine pancreas and not a trace of this diamine can be detected in bovine liver according to FISCHER and BOHN (1957)

Considerable amounts of the diamines putrescine and cadaverine however were found in the developing chick embryo During the embryonic development the concentrations of these showed peaks preceding those of spermidine and spermine but thereafter decrea ed steadily until hatching Only trace amounts of these compounds could be found in 4 day old chicks (CALDARERA BARBIROLI and MORUZZI 1965) Some evidence indicating the presence of putrescine in the chick embryo has also been obtained by RAINA (1963)

Intracellular distribution of putrescine spermidine and spermine Putrescine spermidine and spermine as cations have a high affinity for various acidic compounds occurring within bacterial or animal cells This phenomenon is precisely very interesting in living cells but unfortunately it also complicates the determination of the intracellular distribution of these amines since after disintegration of the cell secondary redistribution can take place The diamines

and polyamines are thus after homogenization of the tissue rapidly taken up by various particulate fractions of the cell (TABOR and TABOR 1964)

COHEN and LICHTENSTEIN (1960) found that the ribosomal fraction of E coli contained putrescine and spermidine in amounts equivalent to approximately 15 per cent of their total content in this bacterium Further they concluded that ribosomal putrescine which was the main amine and spermidine occurred in a nonexchangeable form and were not derived from the extracting medium However according to TABOR and KELLOG (1967) the polyamine content of E coli ribosomes is dependent on the nature and concentration of the ions in the medium used for their preparation

The molar amounts of putrescine and spermidine have been calculated to be sufficient to neutralize about 8 per cent of the ribosomal ribonucleic acid phosphate in E coli (COHEN and LICHTENSTEIN 1960) According to ZILLIG KRONE and ALBERS (1959) the amine content of E coli mainly putrescine and cadaverine in this study could neutralize at least a third of the acidic groups of the ribosomal ribonucleic acid The presence of the diamine cadaverine in the ribosomal particles of E coli has also been stated by SPAHR (1962) The total diamine and polyamine content amounted to 0 4 per cent of the dry ribosome and the amines were found approximately in a molar ratio putrescine/spermidine/cadaverine,3 2 1 Only traces of spermine were detected

If the polyamines in E coli ribosomes at least to some extent are non exchangeable the ribosomes isolated from guinea pig pancreas rapidly take up labelled spermidine from the medium (SIEKEVITZ and PALADE 196.) This also held true for liver homogenate in which exogenously added labelled polyamines were quickly taken up by particulate fractions of the disintegrated cell (TABOR and TABOR 1964)

The subcellular distribution of the polyamines spermidine and spermine in rat liver has recently been studied by RAINA and TELARANTA (1967) About 60 per cent of the total cell polyamines was recovered from the microsomal fraction at a low ionic medium and the percentage distribution of spermidine and spermine was very close to that of ribonucleic acid in isolated subcellular fractions With increasing ionic strength especially in the case of divalent cations the polyamines were partly released from the microsomes into the medium The added labelled polyamines were taken up by particles and again released by increasing the ionic strength

In spite of the possibility of redistribution of polyamines after disintegration of a mammalian cell it seems that in all probability the intracellular polyamines are bound to nucleic acids and according to SIEKEVITZ and PALADE (196.) as well as RAINA and TELARANTA (1967) the binding is due to a salt bond between the amine and ribonucleic acid phosphate

BIOSYNTHESIS

In microorganisms The first experiments demonstrating the biosynthesis of spermidine in E coli and Aspergillus nidulans were carried out by TABOR ROSENTHAL and TABOR (1956 1957 1958) They showed that the

four carbon moiety of the polyamine molecule was derived from putrescine $^{14}C^{15}N$ putrescine was incorporated as a unit into spermidine molecule in growing cultures of E coli and A nidulans Using an ornithine requiring mutant of E coli they also showed that this amino acid could serve as a precursor for putrescine and spermidine synthe is

GREENE (1957) demonstrated that the propylamine unit of spermidine molecule was derived from the amino acid methionine

The biosynthesis of spermidine from putrescine and methionine has also been demonstrated with cell free extract of E coli (TABOR ROSENTHAL and TABOR 1958)

As stated previously ornithine can act as a precursor of putrescine synthesis in E coli indicating that one of the precursors of putrescine is a late intermediate of the arginine biosynthetic pathway GALE (1940) demonstrated an induced ornithine decarboxylase in E coli which in special culture conditions catalyzed the formation of putrescine This enzyme however could not be responsible for the biosynthesis of large amounts of putrescine normally occurring in E coli grown in minimal medium Recently MORRIS and PARDEE (1965 1966) have reported the presence of an other type of enzyme decarboxylating ornithine in E coli They called this enzyme a biosynthetic ornithine decarboxylase to distinguish it from the inducible enzyme described by GALE (1940) This biosynthetic enzyme differed from the latter in its pH optimum heat stability and various other properties The decarboxylation of ornithine to yield putrescine was also catalyzed in cell free preparations (MOR PIS and PARDEE 1965 1966)

Not only ornithine but also arginine was decarboxylated in cultures of E coli The decarboxylation of arginine serves as an other route for putrescine formation in E coli Again the presence of two distinct enzymes for the decarboxylation of arginine is obvious in this organism Like ornithine decarboxylase there is an inducible (GALE 1940) and a biosynthetic (MORRIS and PARDEE 1966) arginine decarboxylase in E coli The conversion of arginine to putrescine involved two distinct reactions decarboxylation yielding agmatine (decarboxylated arginine) and subsequent hydrolysis of the guanidino group of agmatine yielding putrescine and urea as final products (MORRIS and PARDEE 1966) It is unlikely that ornithine is an intermediate in this reaction since the presence of arginase activity in E coli has never been reported (MORRIS and PARDEE 1966)

The formation of spermidine from putrescine and methionine involves first the conversion of methionine to S adenosylmethionine The mechanism for this methionine activation is that previously described for transmethylations (CAN TONI and DURELL 1957) The formation of S adenosylmethionine is followed by a subsequent decarboxylation of this compound The propylamine moiety from the decarboxylated S adenosylmethionine is then transferred to putrescine to yield spermidine (TABOR and TABOR 1964)

Putrescine is also incorporated into spermine (TABOR ROSENTHAL and TABOR 1958) suggesting the possible role of spermidine to act as a precursor of spermine biosynthesis

The various steps involving spermidine biosynthesis in E coli are summarized in Fig 1

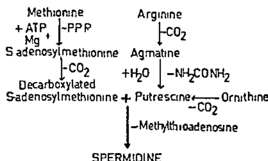


Fig 1 Spermidine synthesis in E coli

In addition to the synthesizing capasity of E coli this bacterium can also take up putrescine and polyamines from the medium The amines are adsorbed by the bacterium as well as actively transported through a time and metabolic processes-dependent system which does not occur at 0° C and is markedly decreased by the omission of glucose (TABOR and TABOR 1966)

In animal tissues The first evidence indicating the biosynthesis of polyamines from putrescine in mammalian tissues was obtained by TABOR ROSENTHAL and TABOR (1956) In a preliminary paper they reported a small incorporation of radioactivity from $^{14}C^{15}N$ putrescine into spermidine in minced rat prostate

RAINA (1962b 1963) demonstrated that the known precursors of spermidine in bacteria i e putrescine ornithine and methionine were also incorporated into spermidine and spermine in developing chick embryos The radioactivity from ^{14}C putrescine or ^{14}C ornithine was rapidly incorporated into spermidine whereas a lag period of several hours was obvious before radioactive spermine could be isolated On the other hand the incorporation of radioactivity from ^{14}C methionine into both polyamines occurred at almost equal rates

Putrescine and methionine (RAINA 1964) as well as the amino acid arginine (JÄNNE and RAINA 1966) have been shown to serve as precursors of polyamines in various rat tissues After the injection of putrescine into young rats the label was again found in the early phase mainly in spermidine whereas the label from methionine was transferred roughly equimolarly into both polyamines (RAINA 1964) These results suggested that in animal tissues spermidine acts as a precursor for spermine synthesis

The role of spermidine in serving as a precursor for spermine formation in animal tissues has later been confirmed indirectly (RAINA JÄNNE and SIIMES 1964) and recently directly (JÄNNE and RAINA 1966 SIIMES 1966 1967)

Interestingly the converse reaction formation of spermidine from spermine has also been demonstrated as occurring in rat tissues (SIIMES 1967)

On the other hand after administration of uniformly labelled ^{14}C glucose or ^{14}C proline to chick embryos no radioactivity could be detected in the polyamines (RAINA 1963) Nor was the label from the uniformly labelled ^{14}C glutamate incorporated into spermidine or spermine isolated from the rat liver (JÄNNE and RAINA 1966)

In addition to the preliminary paper by TABOR ROSENTHAL and TABOR (1956) the synthesis of polyamines has not been reported to occur in animal tissues *in vitro* Recently however the decarboxylation of ornithine spermidine synthesis from putrescine and the interconversion reactions between spermidine and spermine have been demonstrated in Ehrlich ascites cells *in vitro* (SIIMES and JÄNNE 1967)

Little is known of the distribution of putrescine and even less is known of its biosynthesis in animal tissues After administration of ^{14}C ornithine to chick embryos RAINA (1963) could detect a weak ninhydrin positive fraction which contained radioactivity This fraction showed a mobility similar to putrescine in paper electrophoresis and paper chromatography The observation suggests that in animal tissues too the polyamines spermidine and spermine are synthesized *via* putrescine

DEGRADATION

In addition to the above mentioned interconversion reactions between spermidine and spermine very little is known of the metabolic pathways involving the degradation of these compounds in animal tissues *in vivo* Some additional evidence suggesting the degradation of spermine to spermidine has however been obtained When spermine is injected parenterally into an animal a small part of the dose appears as spermidine in the urine On the other hand when spermidine is administered no spermine is excreted (ROSENTHAL and TABOR 1956)

Using ^{14}C labelled polyamines SIIMES (1967) could demonstrate that spermidine and spermine are convertable into each other in rat tissues Further both polyamines were oxidized into carbon dioxide in the rat *in vivo*

The oxidation of diamines and polyamines in animal tissues by plasma amine oxidase (HIRSCH 1953a 1953b) as well as their oxidative and non oxidative degradation in microorganisms are reviewed in detail by TABOR and TABOR (1964)

SOME ASPECTS OF THE POSSIBLE PHYSIOLOGICAL ROLE OF PUTRESCINE AND POLYAMINES

In microorganisms Many of the effects these amines exert on various cellular and subcellular components could be explained on the basis of their physico-chemical structure The diamines and polyamines are basic substances having a high affinity to the acidic groups of many natural compounds By

considering diamines and polyamines as polycations the stabilizing effects they exert on nucleic acids for instance can be understood In general the effects are in many instances closely related to those observed with histones and protamines as well as divalent cations Mg^{++} and Ca^{++}

One of the first signs suggesting the physiological importance of these compounds was that indicating their requirement for the growth of certain microorganisms i e Hemophilus parainfluenzae (HERBST and SNELL 1949) and some strains of Neisseria (MARTIN PELCZAR and HANSEN 195_)

Various features of the effect of polyamines on nucleic acids have been reported in vivo and in vitro This area of polyamine function however is also reviewed in detail by TABOR and TABOR (1964) but some of the latest investigations will be cited here

The polyamines spermidine and spermine clearly increased the denaturation temperature of deoxyribonucleic acid obtained from Bacillus subtilis (TABOR 1961) Similar stabilizing effects by polyamines have also been observed on ribonucleic acid (MITRA and KAESBERG 1963 GOLDSTEIN 1966)

Polyamines also exert great influence on the reactions involving nucleic acids as substrates Spermine inhibits ribonuclease enzyme in yeast cells (SCHLENK and DAINKO 1966) Spermidine and spermine inhibit the degradation of nucleic acids under various other conditions These effects are however considered to be an indirect inhibition of nucleases based possibly on stabilization of the cells or subcellular particles by polyamines (TABOR and TABOR 1964)

Putrescine spermidine spermine and other related compounds stimulated the synthesis of ribonucleic acid catalyzed by ribonucleic acid polymerase in Azotobacter vinelandii (KRAKOW 1963) This stimulation however depended on the nature of the primer and took place only when the reaction was primed by native deoxyribonucleic acid With synthetic polyribonucleotides as the primer there was even an inhibition of the reaction indicating that the mechanism for this stimulation was not due to inhibition of the nucleases contaminating the polymerase preparation Similar stimulation of ribonucleic acid synthesis by polyamines was also observed in Micrococcus lysodeikticus (FOX and WEISS 1964) Spermine has a stimulatory effect on deoxyribonucleic acid polymerase reaction in Physarum polycephalum (BREWER and RUSCH 1966)

The patterns of cellular spermidine and ribonucleic acid in a polyauxotrophic mutant of E coli changed in a similar manner under various nutritional conditions and the addition of spermidine to a medium devoid the amino acid arginine caused a several fold stimulation in the ribonucleic acid synthesis The elimination of arginine from the medium not only inhibited ribonucleic acid synthesis but also prevented the cellular accumulation of spermidine Putrescine under these conditions was only slightly stimulatory and the stimulatory effect of spermidine on ribonucleic acid synthesis was gradually abolished by increasing the concentration of putrescine in the medium (RAINA and COHEN 1966)

Using an isogenic pair of relaxed and stringent auxotrophic strains of

E. coli. COHEN, HOFFNER, JANSEN, MOORE and RAINA (1967) could further show a close relationship between spermidine and ribonucleic acid synthesis. In amino acid starved cultures the relaxed strain produced greater quantities of both ribonucleic acid and spermidine compared to the stringent strain. The synthesis of spermidine in the relaxed strain was independent of ribonucleic acid accumulation.

The action of putrescine and polyamines on ribosomes and protein synthesis is covered by a relatively well documented area in the literature concerning their physiological function. Many correlations between the effects of putrescine and polyamines and divalent inorganic cations at the cellular and subcellular level have been observed. As stated previously, the ribosomes of both bacterial and animal cells contain considerable amounts of putrescine, spermidine and spermine (COHEN and LICHTENSTEIN 1960, ZILLIG, KRONE and ALBERS 1959, RAINA and TELARANTA 1967). These compounds have been observed to cause similar ribosomal aggregations and stimulation of protein synthesis, as do the divalent cations (HERSCHKO, AMOZ and MAGER 1961, MOLLER and KIM 1965). On the other hand, the completely opposite effect, an inhibition of protein synthesis, has also been reported. Spermine inhibited protein synthesis in Walker 256 carcinosarcoma under the same culture conditions as it stimulated ribonucleic acid synthesis (GOLDSTEIN 1965). Spermine also inhibit polypeptide synthesis in a subcellular system derived from the L 1-10 mouse ascites leukemia (OCHOA and WEINSTEIN 1965) and protein synthesis in growing cells of *Staphylococcus aureus* (FRIEDMAN and BACHRACH 1966). The effects of polyamines on protein synthesis thus seems to be critically dependent on their concentration: low concentrations stimulate and high concentrations inhibit protein synthesis.

Oxidized spermine is toxic to various bacteria, inactivates bacterial and plant viruses (TABOR and TABOR 1964) and inhibits the multiplication of Ehrlich ascites cells (BACHRACH, ABZUG and BEKIERKUNST 1967).

Relationships between polyamines and nucleic acids in animal tissues. Most of the observations indicating connections between polyamines and nucleic acids have been obtained from microbiological material, i. e. from bacteria and viruses. On the other hand, there are only a few, and in most cases indirect studies concerning this relationship in animal tissues. The assumptions suggesting an association between polyamines and nucleic acids, especially with ribonucleic acid, in animal tissues are almost without exception based on parallel changes found in polyamine and nucleic acid concentrations under certain experimental conditions.

The concentration of polyamines showed similar changes to those shown by nucleic acids during the development of the chick embryo (RAINA 1963, CALDARERA, BARBIROLI and MORUZZI 1965). In addition to the polyamines the diamines putrescine and cadaverine also showed increases and decreases preceding those observed in nucleic acids at various developmental stages (CALDARERA, BARBIROLI and MORUZZI 1965). This parallel behaviour between polyamines and nucleic acids was not entirely limited to physiological developmental conditions in the embryo. Some unphysiological experimental

conditions which varied the polyamine pattern also caused similar changes in nucleic acids

As pointed out in previous sections spermidine concentration was at its highest in rat tissues at birth therefater gradually decreasing with age (JÄNNE RAINA and SIIMES 1964) This uniform feature observed in each tissue studied shows some similarities to the changes in nucleic acids especially ribonucleic acid taking place during the postnatal development of the rat (FUJII and KOYAMA 1962 OLIVER BALLARD SHIELD and BENTLEY 1962)

If the polyamines in animal tissues too are in some way related to nucleic acids such a connection is not so obvious during the normal developmental growth of the animal On the other hand this correlation is much more apparent in tissues subjected to extreme experimental conditions e g in rat liver undergoing regenerative growth

When the medial and left lateral lobes of the rat liver have been removed the remaining lobes undergo a process of rapid growth resulting in an almost complete regeneration of the original tissue mass within a few weeks During this period of regeneration many biochemically interesting alterations take place rapidly The first changes occur in the areas of glycogen and lipid metabolism (HARKNESS 1957) and in the biosynthesis of ribonucleic acid Partial hepatectomy of the rat also provokes rapid changes in the field of polyamine metabolism (DYKSTRA and HERBST 1965 RAINA JÄNNE and SIIMES 1965 1966) During the regeneration process spermidine concentration like that of ribonucleic acid increased very sharply reaching a maximum at 64 h after partial hepatectomy There was also early stimulation in the biosynthesis of this polyamine which was indicated by a marked rise in the incorporation of radioactive precursors into spermidine (DYKSTRA and HERBST 1965 RAINA JÄNNE and SIIMES 1965 1966) A clear increase in synthesis occurred as early as 8 h after partial hepatectomy and a net increase in liver spermidine could be seen at 16 h The behaviour of ribonucleic acid content in regenerating liver closely resembled that of spermidine On the other hand the concentration of spermine (per wet wt unit) like that of deoxyribonucleic acid decreased significantly during early regeneration and an increase in their concentration exceeding the control values could not be seen until 9 days after partial hepatectomy (RAINA JÄNNE and SIIMES 1966)

Thus there is an obvious correlation between polyamines and ribonucleic acid in animal tissues too but the exact function of these compounds is almost completely obscure However on the knowledge we have of them in the microbiological world we can assume that polyamines also exert similar stabilizing and neutralizing effects on nucleic acids in animal tissues as they do in bacteria and viruses This hypothesis is strongly supported by the discovery that during the whole period of regeneration the ratio of polyamine nitrogen to nucleic acid phosphate especially ribonucleic acid remained quite constant (RAINA JÄNNE and SIIMES 1966)

There are in addition to regenerating liver some other conditions under which the behaviour of polyamines and nucleic acids closely resemble each other CALDARERA COZZANI and MORUZZI (1966) showed recently that in rat livers subjected to ionizing radiation there was a considerable increase in the

content of both polyamines a lesser increase in ribonucleic acid while deoxyribonucleic acid showed less evident changes

An injection of growth hormone into rats provokes many anabolic effects in the liver tissue including a marked stimulation of ribonucleic acid synthesis (TALWAR PANDA SARIN and TOLANI 1962 KORNER 1963) The content of liver polyamines are also affected by this hormone (KOSTYO 1966) The changes in hepatic polyamine concentrations followed by growth hormone treatment were essentially similar to those observed in regenerating liver spermidine increased but spermine decreased or remained unchanged On the other hand after hypophysectomy the situation was just the reverse spermidine decreased and spermine increased slightly

In this connection the close subcellular distribution of polyamines and ribonucleic acid in rat liver should also be mentioned (RAINA and TELA RANTA 1967)

An interesting effect of these compounds is their ability to serve as growth factors for a mammalian cell line also indicating their importance even for animal tissues (HAM 1964)

On the results of the several investigations cited in this section it could be concluded that there are undoubtable relationships between putrescine spermidine spermine and nucleic acids in microorganisms as well as in animal tissues The simplest explanation of some of these phenomena is that based on the ionic affinity between positively charged polyamines and negatively charged nucleic acids thus resulting in a neutralization between these compounds On the other hand there are also many reports indicating even a primary regulation of nucleic acid metabolism by polyamines

Outline of present study

As stated in previous sections polyamines and diamines are widely distributed not only in microorganisms but also in animal tissues The knowledge we have of their possible physiological role and significance is almost entirely based on studies carried out on microorganisms

A great deal of attention has also been paid to the occurrence biosynthesis and behaviour of these compounds in the microbiological world In recent years however increasing evidence has indicated their importance in animal tissues too On the other hand relatively little is known of their biosynthesis in animal tissues or the association of their biosynthetic pathways with the intermediary metabolism of animals

The polyamines spermidine and spermine have been shown to be synthesized in animal tissues from the same precursors as in microorganisms The amino acids ornithine arginine and methionine belong to the normal constituents of an animal organism Although there are reports of the isolation of putrescine from mammalian tissues it is questionable whether this diamine occurs in sufficient amounts to serve as a natural precursor of polyamines in mammalian organisms too

The behaviour of polyamines in a rapidly growing animal tissue is an interesting feature in their metabolism What is the sequence of the events culminating in a marked increase in the concentration of spermidine in regenerating liver or in hepatic tissue after growth hormone treatment?

The present investigation is divided into three parts

I The occurrence of the diamine putrescine in normal rat liver at different ages and its conversion *in vivo* and *in vitro* into hepatic polyamines in normal and ethionine-treated rats

II The changes taking place in putrescine and polyamine contents during early liver regeneration and the alterations in various biosynthetic steps involving polyamine biosynthesis

III The effect of pituitary growth hormone on putrescine and poly amine contents as well as on their biosynthesis in rat liver

Material and methods

EXPERIMENTAL ANIMALS

The animals used were albino rats of Wistar strain (an inbred strain from this laboratory) or as in the hormone experiments of Sprague Dawley strain (obtained from Oy Orion Ab Mankkaa Finland) The rats were kept on a routine laboratory diet (Hankkija Oy Helsinki Finland) In general the animals were allowed to eat and drink *ad libitum* except those used in the hormone experiments which fasted 24 hours before killing The age which seems to be an important factor affecting the metabolism of polyamines varied in different experiments and will be mentioned separately with each experiment The rats were killed by decapitation before assay procedures

IN VIVO TECHNIQUES

Partial hepatectomy Partial hepatectomy of the rat was performed essentially as described by HIGGINS and ANDERSON (1931) Under light ether anaesthesia the medial and left lateral lobes were removed The removed part of the liver was approximately equal to two thirds of the whole rat liver The mortality rate during or after this relatively simple surgical procedure was minimal No special post operative care was found to be necessary and the animals were allowed to eat and drink *ad libitum* immediately after operation

In preliminary experiments it was observed that the laparotomy or anaesthesia alone had no effect on putrescine and polyamine biosynthesis Thus the control groups of various experiments were represented by unoperated rats From those control animals only the right lateral and caudate lobes (the remaining part after partial hepatectomy) were used for analysis In each experiment the rats were decapitated at the same time the precursors were injected at the same time but the operations were performed 2 to 72 hours before analysis

Treatment with growth hormone The pituitary growth hormone used was a commercial preparation (Somacton Ferring Malmo Sweden) This drug had a relatively high degree of purity containing approximately 1 international unit per 0 5 mg of dry substance (personal communication by Ferring) The hormone was dissolved in a small volume of its original solvent containing 0.250 g glucose 0 025 g phenol and HCl a d pH 4 in 5 ml of distilled water and thereafter diluted 4 fold with 0 9 per cent NaCl The drug was administered intra

peritoneally in a volume of 2 ml The control animals received the solvent only in equal volume and dilution In all experiments the animals were fasted for 24 hours before decapitation

IN VITRO TECHNIQUES

After decapitation of the rats the livers used for in vitro experiments were immediately perfused in situ with cold physiological saline and placed in ice-cold buffer at 3° C.

In a few preliminary experiments tissue slices of rat liver were used The slices were prepared at 3° C with a slicer described by STADIE and RIGGS (1944) The tissue pieces were then weighed and transferred into flasks containing ice cold Krebs Ringer phosphate buffer (Ca^{++} omitted) at pH 7 4 300 or 500 mg of slices were used for each incubation After incubation the slices were washed twice with cold buffer homogenized in 0 1 N HCl and analyzed for the radioactivity in polyamine fractions

In the majority of the preliminary experiments and in all the experiments presented here liver homogenates were used After perfusion and excision the livers were immediately homogenized with a Potter Elvehjem type homogenizer The homogenization medium was usually 250 mM sucrose containing 10 mM potassium phosphate buffer at an initial pH of 7 8 and the homogenates contained 40 to 50 per cent (wt./vol) liver tissue The incubations were performed in 25 ml flasks usually in a volume of 2 ml in an incubator with mechanical shaking The reaction was stopped by adding 4 ml of 0 1 N HCl to the flasks and placing them in ice at 3° C

The blanks were kept in ice at 3° C during the whole period of incubation

To exclude possible bacterial contamination in the incubations a series of liver homogenates was prepared and incubated under sterile conditions These sterile preparations showed essentially similar spermidine synthesizing capacity as did those prepared in the normal manner

ANALYTICAL METHODS

Reagents The reagents used were of analytical grade in most instances obtained from Merck AG (Darmstadt Germany)

Reference tandards Putrescine dihydrochloride spermidine phosphate and spermine tetrahydroclonde were obtained from the Mann Research Laboratories (New York U.S.A) The albumin standard for protein measurements was obtained from Kabi (Stockholm Sweden)

I otopes All the isotopes used were purchased from the New England Nuclear Corporation (Boston Massachusetts U.S.A) The following radioactive precursors of putrescine and polyamines were used DL arginine-5 ^{14}C (in 0 1 N HCl) specific activity 5 0 mC/mmole DL methionine-2 ^{1} C specific activity 4 81 mC/mmole DL-ornithine-5 ^{14}C (in 0 1 N HCl) specific activity 9 8 mC/mmole and putrescine 1 4 ^{1} C (as dihydrocloride) specific activity 9 50 or 5....

mC/mmole *The radioactive stock solutions were stored at* $-20°$ *C and diluted* before use with 0 9 per cent NaCl or with appropriate buffer in *in vitro* experiments The isotopes were administered intraperitoneally in each *in vivo* experiment

Determination of permidine and spermine from tis ue samples The poly amines were determined by the method introduced by RAINA (1963) and later modified by JÄNNE RAINA and SIIMES (1964) The procedure is briefly as follows After homogenization and deproteinization of a tissue sample the polyamines were extracted from a strongly alkaline solution into n butanol The butanol phase was then evaporated to dryness the residue dissolved in a small volume of 0 1 N HCl and stored in a small test tube until use The final separation of the amines was performed by paper electrophoresis using 0 1 M citric acid buffer at pH 3 6 or 4 3 The mean recoveries in the butanol extraction were 92 and 95 per cent respectively for spermidine and spermine The appropriate corrections for polyamines due to loss during extraction were applied

The staining of polyamines with amido black washing procedures and elution of the stained fractions have been previously described (JÄNNE RAINA and SIIMES 1964)

Determination of putrescine Putrescine was determined from the same samples as polyamines but always using 0 1 M citric acid buffer at pH 4 3 in the paper electrophoresis The paper strips were stained with ninhydrin in acetone (RAINA and COHEN 1966) instead of amido black The coloured fractions were then eluted with 6 ml of water ethanol glacial acetic acid 1 4 5 (by vol) containing 2 mg of cadmium acetate per ml The extracted red colour which showed maximum absorption at 505 mμ was found to be completely stable when kept in dark for at least three hours The colour was measured within one hour with a Beckman B spectrophotometer at 505 mμ

The mean recovery of putrescine in the butanol extraction was 86 per cent and the appropriate correction was applied

Protein measurements Proteins were determined from the washed trichloro acetic acid precipitate according to LOWRY ROSEBROUGH FARR and RAN DALL (1951) using human albumin as a reference standard

Radioactivity measurements Putrescine and polyamines were counted di rectly from the electropherograms after staining the strips with 0 1 per cent ninhydrin in ethanol in a Packard Tri Carb liquid scintillation spectrometer (Packard Instrument Company Inc La Grange Illinois U S.A) As a liquid scintillator 2 ml of 0.3 per cent PPO (2,5 diphenyloxazole) and 0 01 per cent dimethyl POPOP [1 4 bis 2 (4 methyl 5 phenyloxazolyl) benzene] (Packard Inst rument Company) in toluene was used

The radioactivity of liver protein was also counted from paper strips The washed trichloroacetic acid precipitate was first dissolved with 0 1 N NaOH Thereafter a sample of 0 1 ml was applied onto a small band of filter paper (Whatman No 1) and counted using the above liquid scintillator with an efficiency essentially equal to that with putrescine and polyamines (about 55 per cent)

Paper chromatography of putrescine and polyamines The identity and purity of the amine fractions obtained from routine electrophoresis was checked

as follows The amines were first separated purified and somewhat concentrated by a preparative paper electrophoresis 0 3 to 0 5 ml of an unknown sample was applied onto a 15×39 cm paper strip and run in electrophoresis in the normal manner After drying the papers the fractions were visualized by drawing a few ninhydrin lines with a micropipette along the migration direction of the amines Thereafter the papers were dried at 105° C and the amine fractions were cut off according to the stained ninhydrin lines The unstained parts of the fractions were then eluted with 10 ml of 0 1 N HCl the eluate was made strongly alkaline and the amines were then extracted into n butanol the butanol phase was evaporated to dryness and the rest was dissolved in a small volume of 0 1 N HCl The purified fractions were identified by paper chromatography using one dimensional ascending or descending techniques The following solvent systems were used

I n butanol acetic acid pyridine water (4 1 1 2)

II n propanol conc HCl water (3 1 1)

III n butanol acetic acid water (2 1 1)

In solvent I and II the ascending technique was used The running time was 8 to 10 hours With solvent III the descending technique and a running time of 36 hours was used The stained spots were then compared with the authentic standards which had undergone a similar purification procedure Only one ninhydrin positive spot could be detected in the chromatograms obtained from a purified electrophoretic fraction The Rf values of the spots corresponded to those of the standards in each studied chromatographic system

The radiopurity of putrescine spermidine and spermine was tested according to the above purification procedure The specific activities of putrescine or polyamines from an unknown sample obtained by a single routine paper electrophoresis were essentially the same after the purification procedure

To exclude the presence of acetylated derivatives of putrescine and poly amines (DUBIN and ROSENTHAL 1960) various samples were subjected to acid hydrolysis To each butanol residue originally dissolved in 0 1 N HCl an equal volume of conc HCl was added the test tubes were sealed and placed in an oven for 12 hours at 105° C Thereafter the samples were evaporated to dryness dissolved in the original volume of 0 1 N HCl and analyzed for their putrescine and polyamine contents No evidence of the presence of acetylated derivatives of putrescine or polyamines was obtained

Statistical procedures The presented values are arithmetic means usually with the ranges or ± standard deviations The significance of the differences between the groups are estimated by Student s t test

Results

I OCCURRENCE OF PUTRESCINE AND ITS CONVERSION INTO POLYAMINES IN RAT LIVER

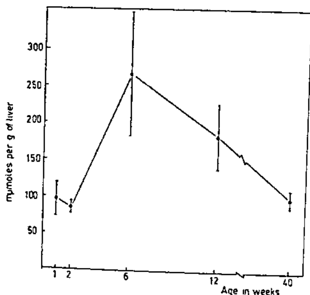
Putrescine, the common diamine in microorganisms can act as a precursor in polyamine biosynthesis also in animal tissues (RAINA 196_b 1963 1964, DYKSTRA and HERBST 1965 JÄNNE and RAINA 1966) This diamine has also been reported as occurring in considerable amounts in developing chick embryos (CALDARERA BARBIROLI and MORUZZI 1965) Although some previous studies have dealt with the occurrence of putrescine in mammalian tissues (WEAVER and HERBST 1958 FISCHER and BOHN 1957) the knowledge we have of the distribution of this compound in mammalian tissues is rather limited Earlier observations indicated that the concentration of putrescine in the pancreas and liver of some mammalian species was very small compared with that of spermidine and spermine These observations question the importance of putrescine in mammalian tissues as a natural precursor in polyamine synthesis or suggest a rapid turnover rate of this compound

The next experiments deal with the occurrence of putrescine and its conversion into polyamines in rat liver under some physiological and experimental conditions

Concentration of putrescine in liver at different ages

Putrescine was found to occur in all the liver samples analyzed even if at a rather low concentration As shown in Fig 2 its concentration varied markedly with age The hepatic concentration of putrescine which during the first two weeks of life was under 100 mμmoles per g wet wt increased sharply thereafter and reached a maximum of _70 mμmoles/g between the first and second month Further increase in age again clearly decreased liver putrescine which in 9 month old rats had diminished to the level found in young rats

It is obvious that in general changes in the concentration of liver putrescine with increasing age do not follow those found in liver polyamines (not tabulated) For example in the above material the concentration of spermidine was at its highest immediately after birth *i e* 1 1_0 mμmoles/g wet wt and then decreased rapidly being only 460 mμmoles at the age



ig 2 The concentration of liver putrescine in relation to age Each age oint represents a mean obtained from five to six male rats except that of the e group 1 which contained five pooled sampl s four organs in each The vertical lines represent stardard deviations of the means

f 40 weeks The concentration of putrescine was thus relatively low ompared with that of spermidine varying from about one tenth to one urd at various stages of development

Conversion of putrescine into polyamines in rat liver in vivo

The biosynthesis of polyamines from exogenously added putrescine in ormal rat liver is demonstrated in Fig 3 The animals used in this periment were two weeks old and received ^{14}C putrescine as an traperitoneal injection 15 to 240 min before analysis As shown in the gure the radioactivity of liver putrescine decreased relatively rapidly hile that of polyamines increased continously Between 15 and 240 min e activity of putrescine expressed as counts/min per g wet wt decreased om 37 100 to 6 700 and that of spermidine increased from 1 600 to 800 respectively The radioactivity of liver spermine however barely ceeded that of the background even at 4 h after th putrescine injection hus between 15 and 240 min the activity of liver putrescine decreased by 400 counts/min per g and that counted in polyamine fractions increased 22 200 counts/min per g, i e at 4 h after the putrescine injection the

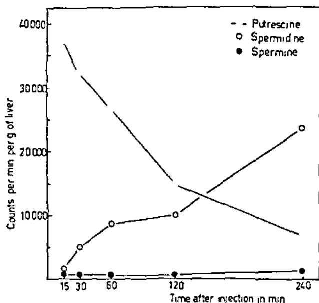


Fig 3 The conversion of putrescine into polyamines in rat liver Two week old male rats received 2 μC (= 0 21 μmole) of putrescine 1,4 ¹⁴C as an intra peritoneal injection Each value represents a mean of three animals

activity of the hepatic polyamines represented about 73 per cent of that having disappeared from the liver putrescine between 15 min and 4 h On the other hand the radioactivity of the total liver polyamines represented only a small portion (2–3 per cent) of the ^{14}C putrescine dose Accordingly it seems that there are other routes for the metabolism of exogenously added putrescine in mammalian organism

An other fact also shown in Fig 3 was the relatively rapid disappear ance of putrescine from hepatic tissue suggesting a rapid turnover rate of this compound This in turn could partly explain the low concentration of putrescine as compared with those of spermidine and spermine whose turnover rates are much slower (JÄNNE and RAINA 1966 SIIMES 1966 1967)

Fate of the radioactivity from exogenously administered ^{14}C putrescine

As pointed out above the radioactivity of the polyamines synthesized from ^{14}C putrescine in rat liver represented only a small percentage of that of the injected isotope It seems thus that there are other routes for the elimination of exogenous putrescine in mammalian tissues

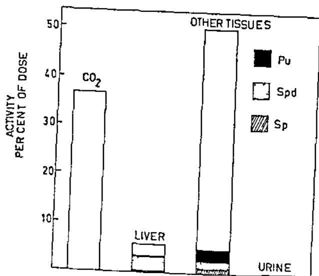


Fig 4 The fate of radioactivity derived from exogenously administered ^{14}C putrescine

Three two month old male rats received 5 μC (= 0 53 μmole) putrescine 1 4 ^{14}C as an intraperitoneal injection 2 h before decapitation Expired ^{14}CO was collected into 4 N NaOH and counted from paper strips The total radioactivity of the liver and other tissues (= rat tissues without liver and skin) represents the activity counted in trichloroacetic acid soluble and insoluble fractions The activities were counted from paper strips ^{14}C putrescine was used as internal standard Pu = putrescine Spd = spermidine Sp = spermine

Fig 4 presents the total distribution of the radioactivity at 2 h after injection with ^{14}C putrescine As shown in the figure over one third of the injected dose was expired as ^{14}CO within 2 hours Only about 6 per cent of the total dose was retained in the liver at 2 h after injection On the other hand more than one half of the activity recovered from the liver was found in putrescine and polyamines mainly in spermidine fraction Most of the radioactivity however was found in the remaining tissues (other tissues except liver and skin) but in comparison with the liver the radioactivity of putrescine and polyamines represented only approximately one tenth of the total radioactivity recovered from those tissues

A very rapid oxidation thus seems to be the major elimination route for exogenously administered putrescine The location of this oxidation is not known nor whether it occurs extracellularly or intracellularly

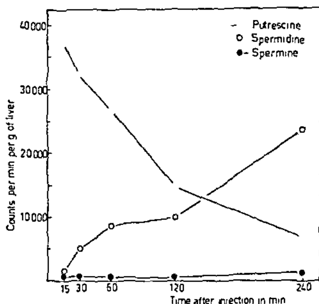


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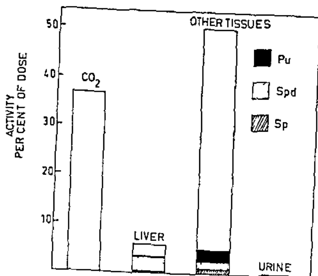


Fig 4 The fate of r dioactivity derived from exogenously administered ^{14}C putrescine

Three two month old male rats received 5 μC (= 0 53 μmole) putrescine 1 4 ^{14}C as an intraperitoneal injection 2 h before decapitation Expired ^{14}CO was collected into 4 N *NaOH* and counted from paper strips The total radioactivity of the liver and other tissues (= rat tissues without liver and skin) represents the activity counted in trichloroacetic acid soluble and insoluble fractions The activities were counted from paper strips ^{14}C putrescine was used as internal standard Pu = putrescine Spd = spermidine Sp = spermine

Fig 4 presents the total distribution of the radioactivity at 2 h after injection with ^{14}C putrescine As shown in the figure over one third of the injected dose was expired as ^{14}CO within 2 hours Only about 6 per cent of the total dose was retained in the liver at 2 h after injection On the other hand more than one half of the activity recovered from the liver was found in putrescine and polyamines mainly in spermidine fraction Most of the radioactivity however was found in the remaining tissues (other tissues except liver and skin) but in comparison with the liver the radioactivity of putrescine and polyamines represented only approximately one tenth of the total radioactivity recovered from those tissues

A very rapid oxidation thus seems to be the major elimination route for exogenously administered putrescine The location of this oxidation is not known nor whether it occurs extracellularly or intracellularly

Table 1 lists the activities of the liver and other tissues expressed as counts/min per g of tissue wet wt As shown the radioactivity in the liver tissue was about twice that in the other tissues In the liver the radioactivity found in spermidine comprised about 40 per cent of the total activity whereas in the other tissues it was only 3 per cent The specific activity of spermidine isolated from the liver was 31 100 counts/min per µmole and that from the other tissues 6 900 respectively The activities without putrescine and polyamines were approximately equal in both hepatic and other tissues i e 38 800 counts/min per g wet wt in the liver and 36 200 in the other tissues

This experiment does not exclude the liver as the place for the oxidation of putrescine but it does somewhat suggest that an important pathway in the hepatic metabolism of putrescine is its conversion into liver polyamines

Table 1 The fate of radioactivity derived from exogenously administered ¹⁴C putrescine See legend for Fig 4

	Activity Counts/min per g wet wt	
	Liver	Other tissues
Total	81 500	40 200
Putrescine	4 800	1 900
Spermidine	34 600	1 300
Spermine	3 300	800

Biosynthesis of spermidine from putrescine in liver homogenates

In the preliminary experiments it was observed that tissue slices of the rat liver or liver homogenate could synthesize spermidine from putrescine or methionine The rate of the biosynthesis of spermidine from putrescine in liver slices was however much slower than that in the intact tissue and also slower than in liver homogenates In general the incorporation of putrescine into spermidine in vitro proceeded without any special additions Neither was the incorporation stimulated

by the addition of glucose adenosine triphosphate or methionine Minor changes in ionic strength did not alter the synthesizing capacity either Two different incubation media were generally used for liver homogenates a) An ionic medium containing 116 mM K^+ 8 mM Na^+ 16 mM Mg^{++} 224 mM Cl 16 mM SO_4^{--} and buffered with 10 mM potassium phosphate buffer at an initial pH of 7 8 thus resembling the physiological intracellular medium b) A crude sucrose medium containing 250 mM sucrose and buffered with 10 mM potassium phosphate buffer at an initial pH of 7 8 Both these media in which the formation of spermidine from putrescine was approximately equal were used in the present study

Fig 5 presents the effect of the incubation time on the incorporation of the label from ^{14}C-putrescine into spermidine in liver homogenates obtained from two-month old rats Accordingly labelled spermidine could soon be isolated from the homogenates incubated in a medium containing radioactive putrescine The radioactivity of spermidine seemed to increase

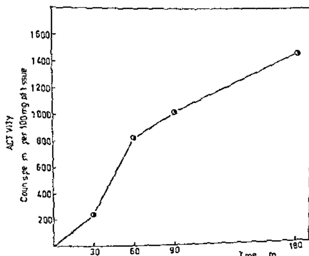


Fig 5 The synthesis of spermidine from putrescine in liver homogenates The homogenate was pooled from three male rats aged two months Each 25 ml flask contained 1 0 g of liver tissue (wet wt) and 0 0168 mM (≈ 0 16 µC/ml) putrescine 1 4 ^{14}C in 250 mM sucrose buffered with 10 mM potassium phosphate buffer at pH 7 8 in a final volume of 2 5 ml Incubation was performed at 37° C using as gas phase atmospheric air and using mechanical shaking The reaction was stopped by adding 4 ml of 0 1 N HCl to the flasks and placing them in ice Each time point represents a mean of three assays

for at least three hours No radioactivity could be detected in spermine fraction under these experimental conditions The rate of conversion of putrescine into spermidine apparently seemed to be slower in the liver homogenate *in vitro* than *in vivo* although the experimental conditions are not directly comparable because of different substrate concentrations According to Fig 5 the total radioactivity per flask found in the spermidine fraction after a 3 hour incubation period represented only about 2–3 per cent of that of the added putrescine The radioactivity found in CO under the same experimental conditions was negligible

As stated in previous seotions (p 10 and Fig .) the age of an animal seems to be an important factor affecting putrescine and polyamine metabolism Fig 6 B illustrates the biosynthesis of spermidine from putrescine in liver homogenates in relation to age The conversion of putrescine into spermidine was relatively slow during the first weeks after birth but then increased rapidly up to the age of 10 weeks On the other hand there was a sudden decrease in spermidine synthesis between 10 and 14 weeks and this slower rate of synthesis seemed to remain until adulthood The changes occurring during the process of aging were quite considerable since more than two fold increase in spermidine biosynthesis from putrescine could be observed between 2 and 10 weeks Thus the changes in spermidine synthesis as measured by the incorporation of putrescine into spermidine in liver homogenates showed some resemblance to the changes in the concentration of putrescine during postnatal development (Fig 2) If this is also valid in an intact organism the accumulation of putrescine in rat liver between 2 and 6 weeks can not be explained in terms of a decrease in the conversion of putrescine into spermidine, and it must be assumed that the biosynthesis of putrescine from its precursors is markedly increased at that time or that the other metabolic routes of putrescine are changed

The above pattern of spermidine biosynthesis from putrescine does not correspond with the concentration curve of spermidine as shown in Fig 6 A In fact the concentration of spermidine decreased continously with age despite the marked increase in its synthesis from putrescine *in vitro* between 2 and 10 weeks The discrepancy between the concentration and biosynthesis of spermidine during early postnatal development could be explained by assuming that the formation of spermine from spermidine is markedly increased during the first weeks after birth This idea is supported by the finding that the concentration of spermine increased from 260 mμmoles/g wet wt to 650 between 2 and 5 weeks *i e* more than two fold

while that of spermidine decreased only from 700 mμmoles/g to 620 at the same time (Fig 6 A)

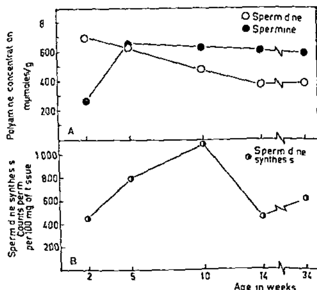


Fig 6 A The hepatic concentrations of spermidine and spermine in relation to age Four pooled samples obtained from three male rats in each group except the 2 week's age group which contained four pooled samples from eight rats
Fig 6 B Spermidine synthesis from putrescine in liver homogenates in relation to age The same material as in Fig 6 A The assay conditions as in Fig 5 except using 0 8 g tissue and 0 0165 mM (= 0 15 μC/ml) putrescine 1,4 ^{14}C in a final volume of 2 ml The incubation time was 60 min Each age point represent a mean of four assays

The early effect of ethionine on the biosynthesis of spermidine

There are some indirect possibilities for the study of the biosynthetic sequences of polyamine metabolism As stated previously methionine can act as a precursor of polyamine synthesis even in animal tissues This amino acid is first converted into S adenosylmethionine at least in the microorganisms and thereafter decarboxylated to form the propylamine moiety for spermidine or spermine molecule Ethionine an ethyl analogue of methionine causes a series of metabolic alterations in animal tissues (STEKOL 1963) Many of these effect can be attributed to the formation of S adenosylethionine (MODY BULBA HOLOWECKY and STEKOL 1963 RAINA JÄNNE and SIIMES 1964) and the marked decrease in tissue adenosine triphosphate level (SHULL 1962 BARTELS and HOHORST

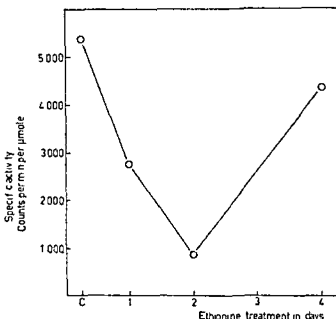


Fig 7 The effect of ethionine on spermidine synthesis from putrescine in rat liver in vivo The animals were one month old male rats receiving daily 0 1 mmole of DL ethionine as an intraperitoneal injection Analysis 3 h after injection with 1 5 µC (= 0 10 µmole) of putrescine 1 4 ¹⁴C Three animals in each group

1963) Ethionine also causes considerable changes in liver polyamines in chick embryos (RAINA 1963) as well as in the rat (RAINA JÄNNE and SIIMES 1964) Ethionine treatment first caused a decrease in liver spermidine and spermine followed by a continued decrease in spermine and marked increase in spermidine concentration Considering that methionine serves as the precursor for polyamines this early decrease in spermidine concentration could be explained by the formation of S adenosylethionine and a decrease in the level of S adenosylmethionine in the tissues The later accumulation of spermidine and simultaneous decrease in spermine concentration might be due to a persistent block between spermidine and spermine (RAINA JÄNNE and SIIMES 1964 SIIMES 1967) at the same time as the synthesis of spermidine is resumed

Fig 7 illustrates the marked effect of ethionine treatment on the biosynthesis of spermidine from putrescine in rat liver *in vivo* After two days treatment with an ethionine dose of 0 1 mmole/animal per day a drastic decrease in the incorporation of radioactivity from ^{14}C putrescine

into spermidine was obvious The specific activity of liver spermidine decreased from the control value 5 400 counts/min per µmole to 770 at 2 days i e by almost 90 per cent and thereafter again increased towards the control value at 4 days after the beginning with the ethionine treatment In this context it should be mentioned that no additional increase in incorporation exceeding the control value could be observed by employing a longer observation period or a larger dose of ethionine It seems thus that the inhibition of spermidine synthesis after ethionine treatment was only transient and the synthesis was completely resumed within a few days This behaviour in spermidine synthesis after ethionine treatment suggests that S adenosylmethionine acts with putrescine as the precursors of spermidine in animal tissues too and that the reduction of this synthesis during the first days of ethionine treatment is due to the formation of S adenosylethionine which probably not can serve as a three-carbon moiety for spermidine ynthesis On the other hand the ability of

Table . Effect of methionine and adenosine triphosphate (ATP) on the incorporation of label from ¹⁴C putrescine into spermidine in liver homogenates obtained from saline and ethionine treated rats

The animals were three month old female rats Two of them received an intraperitoneal injection of physiological saline and two 0 625 mmole DL ethionine 3 h before incubation Each 25 ml flask contained 1 0 g of liver tissue (wet wt) 116 mM K+ 16 mM Mg++ 8 mM Na+ 124 mM Cl 16 mM SO₄-- 10 mM potassium phosphate buffer at pH 7 8 0 011 mM (=0 1 µC/ml) putrescine 1 4 ¹⁴C and additions as indicated in a final volume of 2 ml Incubation time was 60 min. Other conditions as in Fig 5 The values are means ± standard deviations (SD) of three assays

Pretreatment	Additions	Spermidine activity Counts/min per 100 mg of tissue	Change %
Saline	—	490 ± 94	± 0
Saline	DL methionine 0 2 µmole	445 ± 23	− 9
Saline	ATP 2 0 µmoles	410 ± 80	−16
Ethionine	—	170 ± 38	± 0
Ethionine	DL methionine 0 2 µmole	215 ± 12	+26
Ethionine	ATP 2 0 µmoles	230 ± 4	+35

S-adenosylethionine to be incorporated into the spermidine molecule at least after longer treatment is not excluded

Table 2 attempts to show directly a possible lack of available methionine and adenosine triphosphate in the livers of rats treated with ethionine As shown in the table pretreatment with ethionine also caused a marked decrease in the capacity of liver homogenates to synthesize spermidine from ^{14}C putrescine As early as 3 hours after a relatively large single dose of ethionine (0 6 mmole/animal) the incorporation of radioactivity from putrescine into spermidine was decreased from 490 counts/min per 100 mg (wet wt) of hepatic tissue to 170 The addition of methionine or adenosine triphosphate to liver homogenates obtained from normal rats did not increase spermidine synthesis A slight increase in spermidine synthesis was evident when adenosine triphosphate was added to homogenates obtained from ethionine-treated rats This increase however was not very large and the control value was not nearly reached

In an other experiment (not tabulated) it was observed that an addition of methionine and ethionine alone or together to the incubation medium did not alter the spermidine synthesis in liver homogenates *in vitro*

In any case these experiments indicate that the formation of S adenosylethionine and subsequently the lack of S adenosylmethionine cause an early decrease in the biosynthesis and concentration of spermidine in ethionine-treated rats

Concluding remarks

All the above data are in agreement with the idea that putrescine also serves as the natural four carbon precursor in polyamine biosynthesis in rat liver Its concentration is rather low as compared with that of poly amines but this fact can be explained in terms of the rapid turnover rate of putrescine in mammalian tissues The fate of the radioactivity derived from *intraperitoneally administered* ^{14}C *putrescine indicated that in all* probability the most marked route in the metabolism of exogenous putrescine is its oxidation to carbon dioxide but is also seemed that a considerable proportion of the putrescine reaching the liver tissue is conver ted into polyamines at least when compared with other tissues

There is also indirect evidence that the incorporation of the propylamine moiety from methionine occurs *via* the formation of S adenosylmethionine Thus the mechanism for spermidine biosynthesis in rat liver seems to be comparable to that earlier observed in microorganisms

II BIOSYNTHESIS OF PUTRESCINE AND POLYAMINES IN REGENE RATING RAT LIVER

After the removal of a portion of the mammalian liver a series of morphological and biochemical changes occur in the remaining liver These changes finally result in the reconstitution of the original tissue mass

Among the earliest changes taking place after partial hepatectomy are those involving polyamine metabolism The synthesis of spermidine from ^{14}C methionine was markedly stimulated as early as 4 to 8 hours after operation being at that time several times higher than that of the sham operated controls (RAINA JÄNNE and SIIMES 1965 1966) An increase in the concentration of hepatic spermidine could be observed within the first days after operation while that of spermine decreased slightly during that period The incorporation of putrescine into spermidine during the early period of regeneration was somewhat peculiar Like methionine this diamine has been reported to be incorporated more rapidly into spermidine almost immediately after partial hepatectomy (DYKSTRA and HERBST 1965) On the other hand it seemed in our previous study that the incorporation of radioactivity from ^{14}C putrescine into spermidine even decreased during the first day of regeneration (JÄNNE and RAINA 1966)

The following paragraphs attempt to give more detailed picture of the changes in the biosynthetic pathway of spermidine resulting in an almost two fold increase in the concentration of this polyamine a few days after partial hepatectomy

The contents of putrescine and polyamines in rat liver after partial hepatectomy

Table 3 lists the changes in the concentrations of putrescine spermidine and spermine in regenerating rat liver over a period of three days The animals in this experiment were relatively young and as seen in the control animals the concentration of liver putrescine represented about one tenth of that of spermidine A marked accumulation of putrescine took place almost immediately after partial hepatectomy At 4 h after the operation the concentration of putrescine was already more than twice the control value This high level persisted until 12 h after which a gradual decrease took place The concentration of this diamine however clearly exceeded the control value even at 72 h after operation The changes in the

Table 3 The concentrations of putrescine, spermidine and spermine in regenerating rat liver. The animals were one month old female rats, five to six animals in each group. Means ± standard deviations (SD) are presented. C = unoperated controls. Pu = putrescine, Spd = spermidine, Sp = spermine

Time after operation (h)	mμmoles/g (wet wt)			Molar ratios	
	Putrescine	Spermidine	Spermine	Pu/Spd	Spd/Sp
C	133 ± 28	1 110 ± 78	973 ± 53	0.12	1.14
4	294 ± 38 ***	1 130 ± 48	976 ± 56	0.26	1.16
8	272 ± 21 ***	1 220 ± 166	997 ± 41	0.22	1.23
12	282 ± 26 ***	1 290 ± 71 **	822 ± 53 **	0.22	1.57
24	220 ± 38 **	1 440 ± 266 *	785 ± 112 **	0.15	1.84
48	212 ± 21 ***	1 840 ± 111	719 ± 60 ***	0.12	2.55
72	180 ± 35 *	1 890 ± 121 ***	772 ± 67 ***	0.10	2.44

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ The significance of the differences as compared with the controls.

concentrations of spermidine and spermine were in agreement with earlier investigations, i.e. an increase in the concentration of spermidine could be seen at 12–24 h after partial hepatectomy while the concentration of spermine at that time was decreased.

The molar ratio of putrescine to spermidine (Pu/Spd) and that of spermidine to spermine (Spd/Sp) are given in the right hand column of Table 3. The ratio of Pu/Spd increased from the initial value of 0.12 to 0.26 at 4 h and was again normalized at 48 h after partial hepatectomy. A more than two-fold increase could also be observed in the ratio of Spd/Sp but this rise occurred later reaching a maximum of 2.55 two days after partial hepatectomy. Some similarities between the regeneration process and physiological aging can be seen with respect to the Spd/Sp ratio. Accordingly the molar ratio of spermidine to spermine was high in young animals but then decreased rapidly with increasing age (JÄNNE, RAINA and SIIMES 1964).

As shown in Fig. 8 the total putrescine content of the residual liver reached a maximum at 4–12 h postoperatively and thereafter remained essentially unchanged up to 3 days. The accumulation of putrescine in the liver was very rapid since the total amount increased from 200 mμmoles

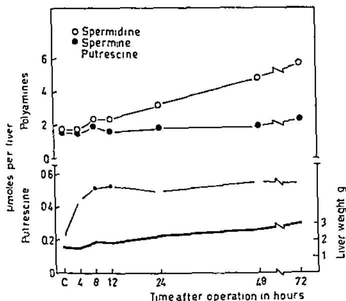


Fig 8 Total putrescine and polyamine contents in regenerating liver The animals were the same as in Table 3 The values are expressed as µmoles per residual liver (wet wt) The thick solid line represents the liver weight curve C = unoperated controls

to 440 during the first 4 hours A clear increase in the spermidine content could be seen at 24 h and in general the rise was more gradual than that seen in putrescine The total spermidine content per residual liver increased over three-fold *i e* from 1 700 mµmoles to 5 650 during the period of three days while that of spermine remained practically unchanged during the whole observation period in spite of a marked increase in liver weight (the thick solid line in Fig 8)

Synthesis of putrescine from ^{14}C ornithine in regenerating liver

What is the origin of the accumulated putrescine after partial hepatectomy? This putrescine might be derived from the intestine and the increased hepatic concentration of putrescine could depend on the decreased detoxication capacity of the regenerating liver However as shown in Fig 9 it is obvious that the biosynthesis of putrescine from ornithine is greatly

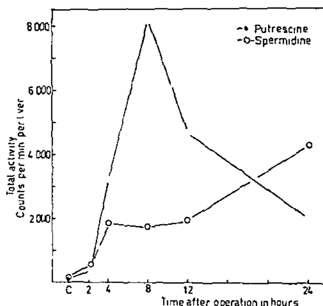


Fig. 0. Incorporation of radioactivity from ¹⁴C ornithine into putrescine and spermidine after partial hepatectomy. 1.5 month old female rats received 4 μC (= 0.41 μmole) of DL ornithine 5 ¹⁴C 2 h before analysis. The activities are expressed as counts/min per residual liver (wet wt.). Three to four animals in each group. C = unoperated controls.

enhanced during the very early period of regeneration. The radioactivity of the putrescine fraction 2 hours after injection with ¹⁴C-ornithine which in the normal liver was 130 counts/min per residual liver, thus barely exceeding that of the background, increased to about 8 000 counts/min at 8 h postoperatively. Thereafter the activity decreased relatively rapidly, still exceeding the control value several times at 24 h after partial hepatectomy. The radioactivity from ¹⁴C ornithine was also incorporated into hepatic spermidine and this incorporation increased several fold after partial hepatectomy too. Some interesting features, however, are seen in the biosynthesis of spermidine from ornithine during early liver regeneration. The increase in the radioactivity of liver spermidine was parallel to that of putrescine only for the first 4 hours after operation, thereafter the radioactivity of the spermidine remained essentially unchanged until 12 h in spite of the considerable increase in putrescine activity at that time. On the other hand, at 24 h postoperatively the total spermidine radioactivity derived from ¹⁴C-ornithine clearly exceeded that of putrescine

Table 4 Incorporation of radioactivity from ¹⁴C ornithine into liver putrescine and spermidine after partial hepatectomy Specific activities are presented as counts/min per µmole (ranges in parentheses) See legend for Fig 9 C = unoperated controls

Time after operation (h)	Specific activity	
	Putrescine	Spermidine
C	1 300 (260—2900)	110 (80—130)
2	2 700 (1100—4000)	370 (310—410)
4	11 100 (8200—14600)	900 (660—1100)
8	16 600 (14800—18900)	1 000 (800—1400)
12	13 900 (9900—17300)	850 (710—870)
24	8 100 (6900—9400)	1 300 (1000—1500)

Table 4 gives the specific activities of putrescine and spermidine obtained from the same experimental animals as in Fig 9 Assuming that the incorporation of radioactivity from ^{14}C ornithine into hepatic spermidine occurs *via* putrescine one can see that despite a marked increase in the specific activity of the precursor practically no additional activity was incorporated into spermidine between 4 and 12 h after operation Only at 24 h postoperatively the specific activity of spermidine was clearly increased while that of putrescine had already decreased from its maximum

Based on the above data it may be concluded that the accumulation of putrescine in the liver during early regeneration at least partly depends on a considerable enhancement of the biosynthesis of this diamine from ornithine It also seems evident that the incorporation of radioactivity from ^{14}C ornithine into spermidine greatly increases during early regeneration and in all probability this incorporation occurs *via* putrescine

Synthesis of spermidine from ¹⁴C putrescine in regenerating liver

To study the biosynthesis of polyamines from putrescine in more detail a group of rats was subjected to partial hepatectomy and ^{14}C putrescine was administered as an indicator of the biosynthesis of polyamines

Fig 10 shows the incorporation of radioactivity from ^{14}C putrescine into spermidine expressed as counts/min per residual liver and also shows the specific activities of putrescine at appropriate stages of early regeneration

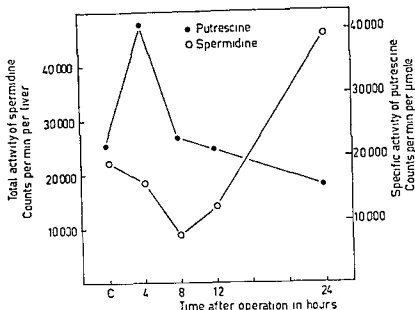


Fig 10 Incorporation of radioactivity from ^{14}C putrescine into spermidine and the putrescine retention in liver after partial hepatectomy Two month old male rats received 3µC (=0 3– µmole) of putrescine 1,4 ^{14}C 2 h before analysis The spermidine activities are expressed as count /min per residual liver (wet wt) the putrescine ones as counts/min per µmole Two to three animals in each group C = unoperated controls

The total spermidine activity decreased as shown in the figure from the control value 22 000 counts/min to 8 100 at 8 h after partial hepatectomy and then again increased being 45 600 at –4 h postoperatively On the other hand except for a peak at 4 h the specific activity of putrescine remained relatively constant during the whole observation period Thus the decreased activity of spermidine after injection of ^{14}C-putrescine into partially hepatectomized rats can not simply be explained by the isotope diluting effect of markedly increased endogenous putrescine

Table 5 gives the corresponding specific activities of spermidine and spermine The specific activities of spermidine are comparable to those observed in total activities except that the increase shown in total activities at 24 h was not so marked since the liver weight and the concentration of spermidine were already increased at that time

If the simple isotope diluting effect of increased endogenous putrescine can not alone cause the marked reduction in the incorporation of label

Table 5 Incorporation of radioactivity from ^{14}C putrescine into liver spermidine and spermine after partial hepatectomy Specific activities are presented as counts/min per µmole (ranges in parentheses) See legend for Fig 10 C = unoperated controls

Time after operation (h)	Specific activity	
	Spermidine	Spermine
C	8 800 (9000 8650)	360 (360 365)
4	7 000 (6000— 8600)	440 (370—580)
8	3 100 (2800— 3500)	260 (230—280)
12	4 600 (3700 5450)	300 (280 325)
24	9 200 (6100—11700)	500 (370—590)

from exogenous ^{14}C putrescine into spermidine during early regeneration there are however other ways of explaining this phenomenon.

The production of putrescine during early regeneration was increased several fold and there can be a preferential use of this endogenous putrescine for spermidine synthesis Thus the exogenously added ^{14}C-putrescine should not be equilibrated with the endogenous putrescine pool This even seems possible since the amounts of ^{14}C putrescine were negligible as compared with the endogenous hepatic pool of putrescine

Table 6 presents the specific activities of putrescine and spermidine during early liver regeneration 2 hours after injection with a very large dose (0 15 mmole/animal) of ^{14}C putrescine This amount of exogenous

Table 6 Incorporation of radioactivity from a large dose of ^{14}C putrescine into liver spermidine and the putrescine retention in liver after partial hepatectomy Two month old male rats received 3 µ (= 0 15 mmole) putrescine 1 4 ^{14}C 2 h before analysis Three animals in each group Specific activities are presented as counts/min per µmole (ranges in parentheses) C = unoperated controls

Time after operation (h)	Specific activity	
	Putrescine	Spermidine
C	37 600	1 480 (1360—1620)
6	33 800	1 380 (1-40—1440)
8	30 000	1 310 (1250—1370)

putrescine was sufficient to increase the concentration of hepatic putrescine 4 to 5 fold Interestingly enough the specific activity of spermidine was essentially unchanged at 6 and 8 h after partial hepatectomy as compared with the controls The specific activity of putrescine also remained relatively constant although a slight dilution of the activity could be seen at 8 h postoperatively Accordingly it seems obvious that the decreased incorporation of label from exogenous ^{14}C putrescine as shown in Fig 10 and Table 5 can be explained in terms of the preferential use of endogenous putrescine and the dilution of the exogenous ^{14}C-putrescine with endogenous putrescine

Spermidine synthesis from ^{14}C-putrescine in liver homogenates obtained from partially hepatectomized rats

Fig 11 illustrates the synthesis of spermidine from ^{14}C putrescine in liver homogenates obtained from normal and partially hepatectomized rats

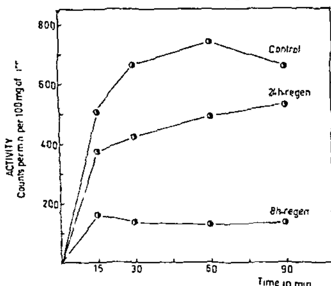


Fig 11 Spermidine synthesis from ^{14}C putrescine in liver homogenates obtained from normal and partially hepatectomized rats

The animals were two-month old male rats Control = pooled homogenate obtained from four unoperated animals 24 h regen = pooled homogenate obtained from 6 rats partially hepatectomized 24 h before incubation 8 h regen = pooled homogenate obtained from 6 rats partially hepatectomized 8 h before incubation Each 25 ml flask contained 0 8 g tissue (wet wt) and 0 0285 mM (0 15 μC/ml) putrescine 1 4 ^{14}C in 250 mM sucrose buffered with 10 mM potassium phosphate buffer at pH 7 8 Other assay conditions as in Fig 5 Each time point represents a mean of three assays

The differences between the homogenates obtained from unoperated animals and from those subjected to partial hepatectomy 8 or 24 h earlier were in general in agreement with those results observed *in vivo* (Fig 10 and Table 5) The incorporation of label from ^{14}C putrescine into spermidine was also greatly depressed *in vitro* in liver homogenates at 8 h after partial hepatectomy The synthesis of spermidine from putrescine *in vivo* seemed to be resumed as early as 24 h after partial hepatectomy and the activities even exceeded those of the control animals at that time However as shown in Fig 11 under these conditions the incorporation curve of this time point ran clearly under the control curve but was still considerably above that observed in 8 h regenerating liver

Thus this experiment in addition seems to support the idea that the synthesis of spermidine from putrescine is somewhat inhibited during early liver regeneration On the other hand the preferential use of endogenous putrescine for spermidine synthesis is not excluded although the liver cells in homogenate are broken and the endogenous and exogenous pools of putrescine could be mixed

Incorporation of radioactivity from ^{14}C arginine into putrescine spermidine and liver total protein after partial hepatectomy

Arginine can also serve as a precursor of polyamine synthesis in rat liver and the incorporation of label from ^{14}C arginine into liver spermidine is markedly increased after partial hepatectomy (JÄNNE and RAINA 1966) In microorganisms this amino acid can even act as the main precursor of putrescine synthesis and is converted into putrescine *via* agmatine (MORRIS and PARDEE 1966)

To study the biosynthetic pathway involving the formation of spermidine from arginine in more detail both hepatic putrescine and spermidine were analyzed for their radioactivity after the injection of normal and partially hepatectomized rats with ^{14}C arginine

As shown in Fig 12 a marked stimulation of the biosynthesis of putrescine and spermidine from arginine was obvious within a few hours after partial hepatectomy The total hepatic putrescine activity expressed as counts/min per residual liver increased from about 100 counts/min to 1 300 at 8 h postoperatively Between 8 and 12 h the putrescine activity again rapidly decreased but still clearly exceeded the initial value at 24 h A similar several fold stimulation was also observed in the incorporation of radioactivity from ^{14}C arginine into spermidine during liver regeneration

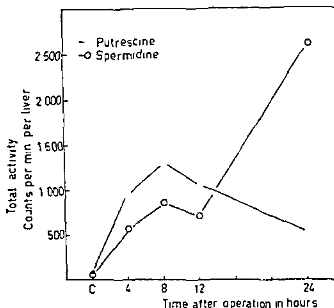


Fig 12 Incorporation of radioactivity from ^{14}C arginine into putrescine and spermidine after partial hepatectomy 1 5 month old female rats received 5 μC (= 1 0 μmole) of DL arginine 5 ^{14}C 3 h before analysis The activities are expressed as counts/min per residual liver (wet wt) Three to four animals in each group C = unoperated controls

The total activity of liver spermidine increased from the control value 50 counts/min to 2 600 at 24 h postoperatively In general the shapes of the incorporation curves of both putrescine and spermidine closely resembled those observed after the administration of ^{14}C ornithine to partially hepatectomized rats

Table 7 represents the corresponding specific activities of hepatic putrescine and spermidine at various stages of the regeneration process The specific activities behaved as did the total activities of putrescine and spermidine

The above results suggested that at least a considerable part of the transfer of label from ^{14}C arginine into spermidine in rat liver occurs *via* putrescine and thus the diamine putrescine serves as an intermediate in the formation of spermidine from arginine What kind of intermediates and reactions are involved in the biosynthesis of putrescine from arginine is not yet clear The bacterial putrescine is formed directly from ornithine or from arginine *via* agmatine (MORRIS and PARDEE 1965 1966) Both of these mechanisms can also exist in mammalian liver but it is possible that

Table 7 Incorporation of radioactivity from ¹⁴C arginine into liver putrescine and spermidine after partial hepatectomy Specific activities are presented as counts/min per µmole (ranges in parentheses) See legend for Fig 1. C = unoperated controls

Time after operation (h)	Specific activity	
	Putrescine	Spermidine
C	620 (260– 9[illegible]0)	40 (20– 60)
4	3 000 (2700–3200)	400 (400– 430)
8	4 700 (3800–5500)	550 (420– 700)
12	4 100 (2900–5300)	300 ([illegible]50– 510)
24	2 200 (1500–2500)	800 (610–1000)

a part of the putrescine formed from arginine is synthesized *via* ornithine The above experiments however did not exclude the possibility that agmatine could act as a direct precursor not only for putrescine synthesis but also for spermidine formation in rat liver

The total amount of the amino acid arginine incorporated into liver

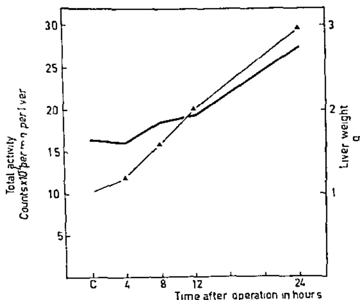


Fig 13 Incorporation of radioactivity from ¹⁴C arginine into total liver protein after partial hepatectomy See legend for Fig 1. The thick solid line represents the liver weight curve C = unoperated controls

putrescine and spermidine was very small however as compared with that incorporated into hepatic protein As shown in Fig 13 the incorporation of radioactivity from DL arginine-5 ^{14}C into total liver protein was considerably increased even during the first day after partial hepatectomy The total activity of hepatic protein increased three fold within 24 hours i e from 102 000 counts/min per residual liver to 296 000 at 24 h postoperatively On the other hand the total activity incorporated from ^{14}C arginine into liver putrescine and spermidine was only 3 100 count /min per residual liver at 24 h after partial hepatectomy (Fig 12) i e approximately 1 per cent of the activity found at that time in liver total protein This total hepatic protein did not represent all the proteins synthesized in liver tis ue since the plasma proteins were not included

Table 8 gives the specific activities of liver protein As shown in the table the most marked increase in the specific activity occurred within the first 12 hours after operation and between 12 and 24 h only an additional increase of 5 per cent could be observed

Table 8 Incorporation of radioactivity from ^{14}C arginine into liver total protein after partial hepatectomy Specific activitie are presented as counts/min per mg of protein (ranges in parentheses) See legend for Fig 12 C = unoperated ontrols

Time after operation (h)	Specific activity
C	320 (270—370)
4	410 (350—460)
8	500 (480—500)
12	600 (520—670)
24	630 (550—670)

Effect of putrescine concentration on the incorporation of radioactivity from ^{14}C-methionine into spermidine in liver homogenates

As reported previously (RAINA JÄNNE and SIIMES 1965 1966) the incorporation of label from ^{14}C methionine into spermidine was greatl increased during liver regeneration This stimulation of spermidine synth from methionine began practically immediately after partial

the incorporation pattern of putrescine was quite different to that of methionine On the other hand in bacteria and bacterial preparations both these compounds act as natural precursors of spermidine synthesis being incorporated equimolarly into this polyamine It also seems apparent that the same mechanism is involved in the natural formation of spermidine in animal tissues In the regenerating liver the synthesis of spermidine from methionine was stimulated several fold immediately after operation contrary to the unchanged or even decreased incorporation of label from ^{14}C putrescine into spermidine at the same time Assuming that the formation of spermidine from ornithine and arginine occurred *via* putrescine the incorporation patterns of these amino acids into spermidine represent the real incorporation of endogenous putrescine into spermidine Accordingly it must be assumed that the decreased activity of spermidine after the injection of ^{14}C putrescine into partially hepatectomized rats could depend on the preferential use of endogenous putrescine and the isotope diluting effect of the increased endogenous putrescine

An indirect attempt was made to also explain the stimulation of spermidine synthesis from ^{14}C methionine during liver regeneration *in vivo* The incorporation of radioactivity from ^{14}C methionine is a very slow reaction *in vivo* and biosynthesis of spermidine from ^{14}C-methionine *in vitro* without any additions is barely detectable Fig 14 presents the incorporation of radioactivity from methionine into spermidine in a liver homogenate obtained from *normal* rats Without the addition of unlabelled putrescine the synthesis of spermidine from methionine under these conditions was minimal With increasing concentrations of putrescine the specific activity of spermidine rose sharply *i e* from 150 counts/min per µmole without putrescine addition to about 1 600 when the medium contained 0 2 mM of DL methionine-2 ^{14}C and 0 2 to 0 3 mM of unlabelled putrescine A similar increase in spermidine activity could be observed by using 0 1 mM of ^{14}C methionine and increasing the concentration of unlabelled putrescine It should be mentioned that the homogenate at zero point of the abscissa also contained their own endogenous putrescine approximately representing a concentration of 0 1 mM Thus the sharpest rise in the specific activity of spermidine took place when the physiological putrescine concentration in relation to the enzyme was doubled This experiment could serve as a possible explanation for the enhancement of spermidine synthesis from methionine during liver regeneration The intracellular putrescine concentration increased just over two-fold within a few hours after partial hepatectomy and this accumulated putrescine fur

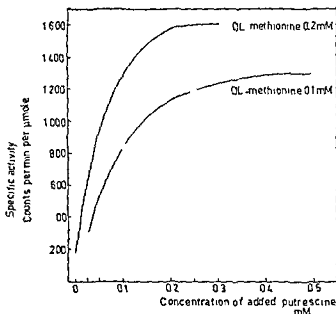


Fig 14 Spermidine synthesis from ¹⁴C methionine in liver homogenates obtained from two month old female rats

Each 25-ml flask contained 1 0 g of tissue (wet wt) 116 mM K^+ 16 mM Mg^{++} 8 mM Na^+ 124 mM Cl 16 mM SO_4^{--} buffered with 10 mM potassium phosphate buffer at pH 7 8 0 1 or 0 2 mM DL methionine 2 ^{14}C (0 5 or 1 0 µC/ml) and unlabelled putrescine as indicated The final volume was 2 ml The incubation time was 60 min Other conditions as in Fig 5 Each point represents a mean of three assays

ther could increase the incorporation of methionine into spermidine On the other hand the specific activity of spermidine was also increased by raising the concentration of ^{14}C methionine from 0 1 to 0 2 mM as shown in Fig 14 The concentration of liver free methionine however, has been reported to remain unchanged during regeneration (FERRARI and HARKNESS 1954) If correct this hypothesis means that the biosynthesis of spermidine from endogenous putrescine is really increased to the same extent as from methionine after partial hepatectomy and the use of exogenous ^{14}C putrescine differs from that of the endogenous one Accordingly the incorporation of radioactivity from ornithine or arginine into spermidine could present the actual spermidine synthesis from putrescine and those incorporations were in fact markedly increased after partial hepatectomy

Concluding remarks

Fig. 15 summarizes the events involving putrescine and polyamine biosynthesis in regenerating rat liver In normal livers (Fig 15 A) the synthesis of putrescine from ornithine is rather slow On the other hand exogenous putrescine is relatively rapidly converted into spermidine and further into spermine The concentration of putrescine is low as compared with that of polyamines At 8 h after partial hepatectomy (Fig 15 B) the situation is changed The incorporation of radioactivity from ^{14}C ornithine into putrescine in increased several fold, the synthesis of spermidine from exogenous putrescine is somewhat inhibited or at least not stimulated and an accumulation of putrescine takes place At that time the conversion of arginine into putrescine had also reached a maximum The concentration of spermidine is not essentially changed At 24 h after operation the situation is again changed (Fig 15 C) The conversion of ornithine into putrescine is still more rapid than in normal liver but is beginning to decrease The formation of spermidine from exogenous putrescine is again restored to its initial level or exceeds it The concentration of putrescine has already decreased from its maximum

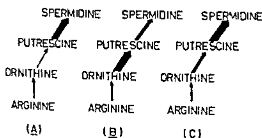


Fig 15 *Spermidine biosynthesis in rat liver after partial hepatectomy*
(A) Intact liver
(B) At 8 h after partial hepatectomy
(C) At 24 h after partial hepatectomy

Very little is known of the conversion of arginine into ornithine during liver regeneration but some recent studies have indicated that at least the activity of the enzyme catalyzing this reaction in the mammalian liver remains practically constant during the first day of regeneration (RAINA, JÄNNE and SIIMES ...

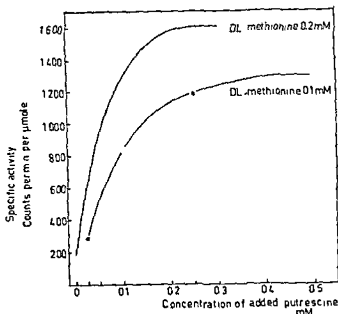


Fig 14 Spermidine synthesis from ^{14}C methionine in liver homogenates obtained from two month old female rats

Each 25 ml flask contained 1 0 g of tissue (wet wt) 116 mM K^+ 16 mM Mg^{++} 8 mM Na^+ 124 mM Cl 16 mM SO_4^{--} buffered with 10 mM potassium phosphate buffer at pH 7 8 0 1 or 0.2 mM DL methionine 2 ^{14}C (0 5 or 1 0 µC/ml) and unlabelled putrescine as indicated The final volume was 2 ml The incubation time was 60 min Other conditions as in Fig 5 Each point represents a mean of three assays

ther could increase the incorporation of methionine into spermidine On the other hand the specific activity of spermidine was also increased by raising the concentration of ^{14}C methionine from 0 1 to 0 2 mM as shown in Fig 14 The concentration of liver free methionine however has been reported to remain unchanged during regeneration (FERRARI and HARKNESS 1954) If correct this hypothesis means that the biosynthesis of spermidine from endogenous putrescine is really increased to the same extent as from methionine after partial hepatectomy and the use of exogenous ^{14}C putrescine differs from that of the endogenous one Accordingly the incorporation of radioactivity from ornithine or arginine into spermidine could present the actual spermidine synthesis from putrescine and those incorporations were in fact markedly increased after partial hepatectomy

Hepatic putrescine and polyamine concentrations after a singl injection of growth hormone into normal rats

The effect of the growth hormone on the conc ntrations of putrescine and polyamines is presented in Table 9 The animals in this experiment received a relatively large dose of growth hormone (4 international units Scmacton Ferring) as an intraperitoneal injection and the time cour e of its action was determined As shown in the table the concentration of putrescine remained relatively unchanged during the whole period of observation At 3 to 6 hours after the hormone injection however a slight but not significant increase in putrescine concentration was observed The changes in the spermidine concentration were not much clearer but a significant increase in the concentration of this polyamine could be observed at 12 h after the hormone injection On the other hand the concentration of spermine remained quite constant The molar ratios of these amines are also given in Table 9 Some minor changes can be seen in both ratios Pu/Spd and Spd/Sp during the observation period i e the ratio of Pu/Spd was slightly increased at 3 h and that of Spd/Sp at 1_ h after injection The changes in the concentrations as well as in the molar ratios were not nearly so marked as in the regenerating liver but in any case some similarities could be seen between these two situations

Table 9 Concentrations of putrescine ~~ermidine and ~ rmine in rat liver after growth hormone treatm nt On mo ith old male r it re eive l an intraperitoneal injection of 4 international units of growth ho mo ie (Som icton) M ans ± standard deviations (SD) are presented F ve animal in e1 h group C = controls treated with solvent only Pu = putre cine Sp l = spermi line Sp = permine

Time after injection (h)	mμmoles/g (wet wt)			Molar ratios	
	Putrescine	Spermidine	Spermine	Pu/Spd	Spd Sp
C	259 ± 24	1 170 ± 178	1 290 ± 91	0 __	0 91
3	_77 ± 31	1 090 ± 59	1 240 ± 7_	0._5	0 88
6	274 ± _1	1 240 ± 101	1 _50 ± 78	0.22	0 99
1_	236 ± 23	1 370 ± 37	1 280 ± 34	0 17	1 07

$p < 0\ 05$ The significance of the d fference as compared with the control group

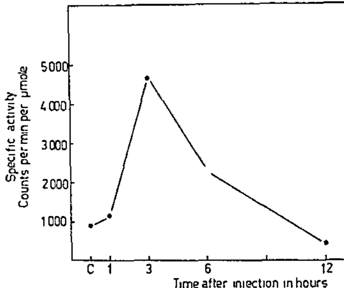


Fig 16 Incorporation of radioactivity from ^{14}C ornithine into putrescine in rat liver after growth hormone injection One month old male rats were treated with a dose of 4 international units of growth hormone (Somacton) 3 µC (= 0 31 µmole) of DL ornithine 5 ^{14}C was injected intraperitoneally 1 h before analysis Each point represents a mean of three animals C = controls treated with solvent only

Effect of growth hormone on putrescine synthesis from ^{14}C ornithine

A slight accumulation of putrescine or spermidine in the livers of growth hormone-treated rats might be due to a retarded degradation of these compounds This possibility was not excluded but as shown in Fig 16 the growth hormone possesses a considerable stimulatory effect on putrescine biosynthesis from ^{14}C-ornithine There was a rapid rise in the specific activity of putrescine derived from ^{14}C-ornithine after a single injection of growth hormone The radioactivity of hepatic putrescine increased from its control value of 900 counts/min per µmole to 4 600 at 3 h after injection with growth hormone and thereafter rapidly decreased to the initial value This stimulation of putrescine synthesis from ornithine seemed to begin practically immediately after the hormone injection and its duration was only a few hours

Although a more than five-fold increase in the biosynthesis of putrescine was obvious only a very slight accumulation, if any of this diamine took place in livers of growth hormone treated rats The reason for this phenomenon should be found in the further metabolism of putrescine

Biosynthesis of polyamines from ^{14}C putrescine after growth hormone treatment

Fig 17 illustrates the biosynthesis of polyamines from ^{14}C putrescine in growth hormone treated rats As shown in the figure a single growth hormone injection was followed by a clear increase in the incorporation of label from ^{14}C putrescine into spermidine The specific activity of hepatic spermidine increased from the initial value of 1 400 counts/min per μmole to 4 600 at 3 h after the growth hormone injection and like that in the formation of putrescine from ornithine this stimulation was also very transitory The spermidine activity again decreased to the control level at 6 h after the hormone injection. The negligible activity of hepatic spermine remained essentially unchanged during the whole period of observation

It seems apparent that the pituitary growth hormone also caused early stimulation of spermidine biosynthesis from putrescine in the hepatic tissue

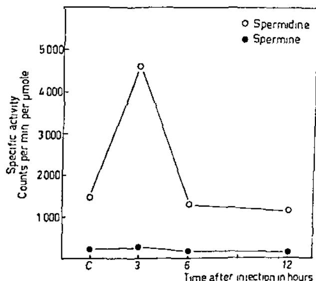


Fig 17 Incorporation of radioactivity from ^{14}C putrescine into hepatic polyamines after growth hormone treatment 4 international units of growth hormone (Somacton) was injected into one month old male rats Analysis 1 h after the injection of 2 μC (= 0 21 μmole) of putrescine 1,4 ^{14}C Each point represents a mean of four to five animals C = controls treated with solvent only

of normal rats Fig 18 gives more detailed information on the mechanism of this stimulation In this figure it is presented part of the putrescine uptake in the liver after the hormone injection, i e the radioactivity counted in liver putrescine spermidine and spermine fractions The upper curve in Fig 18 illustrates the total activity found in hepatic putrescine spermidine and spermine 1 h after injection with ^{14}C putrescine expressed as counts/min per g of liver wet wt The lower curve illustrates the activity counted in the putrescine fraction alone and thus the difference between these two curves represents the polyamines synthesized from ^{14}C-putrescine As shown in the figure the putrescine activity also increased sharply during the first few hours after the hormone injection A maximum over three times the initial value was observed at 3 h and thereafter a rapid decrease was obvious In addition polyamine biosynthesis increased in the same proportion as this putrescine uptake The solid almost horizontal line in Fig 18 represents the percentage proportion of spermidine activity in the activity counted in the total amine fraction As shown this proportion varied within fairly narrow limits i e from 20 to 23 per cent during the

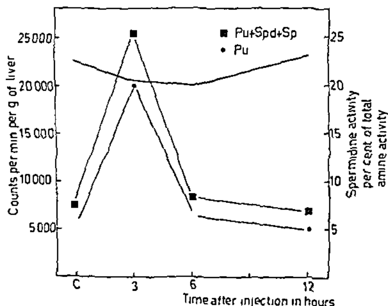


Fig 18 Distribution of radioactivity in liver putrescine and polyamines at 1 h after injection of ^{14}C putrescine into growth hormone treated rats See legend for Fig 17 The solid almost horizontal line represents the percentage of spermidine activity (count/min per g wet wt) of the total amine activity (counts/min per g wet wt in putrescine spermidine and spermine) C = controls treated with solvent only

whole experimental period Thus it seems that real stimulation of spermidine synthesis from putrescine was not necessarily but the increased activity of spermidine after the injection of ^{14}C putrescine into growth hormone treated rats could depend on the markedly increased putrescine uptake of the hepatic tissue

The finding that the growth hormone did not cause real stimulation of spermidine synthesis from putrescine but increased the transport of this diamine into the liver tissue was supported by the discovery indicating that hormone pretreatment did not result in an increase in spermidine synthesis from ^{14}C putrescine in liver homogenates *in vitro* Table 10 presents the synthesis of spermidine from ^{14}C putrescine at 3 h after a single hormone injection As shown in the table no stimulation of spermidine synthesis could be observed under these conditions In liver homogenates the transport of the substrate into the tissue or intact cell is eliminated and the result represents solely the biosynthesis of spermidine from putrescine

In an other experiment (not tabulated) the effect of addition of the growth hormone to the incubation medium was studied Again no differences between the controls and the growth hormone containing incubations could be observed It must be realized however that the conditions employed in these experiments might be inadequate for the action of a hormone as compared with an intact tissue or animal

Table 10 Effect of growth hormone pretreatment on the incorporation of radioactivity from ^{14}C putrescine into spermidine in liver homogenates
The animals were one month old male rats Three of them received 4 international units of growth hormone (Somacton) and three the solvent of the drug 3 h before incubation. Each -5 ml flask contained 0 8 g of liver tissue (wet wt) and 0 0.85 mM (= 0 15 μC/ml) putrescine 1 4 ^{14}C in 250 mM sucrose buffered with 10 mM potassium phosphate buffer at pH 7 8 Incubation time was 60 min Other conditions as in Fig 5 The values are means ± standard deviations (SD) of four assays pooled from three animals

Pretreatment	Spermidine activity counts/min per 100 mg of tissue
Solvent	4.0 ± 53
Growth hormone	380 ± 48

Incorporation of radioactivity from ^{14}C methionine into liver spermidine spermine and total protein after growth hormone treatment

Table 11 presents the incorporation of radioactivity from DL methionine 2 ^{14}C into spermidine spermine and total protein *in vivo* after growth hormone treatment The experimental conditions in this experiment were the same as previously and the activities of the growth hormone-treated rats represents the situation at 3 h after a single hormone injection The specific activity of spermidine increased from 350 counts/min per µmole to 670 *i e* almost two-fold On the other hand that of spermine merely decreased after the hormone injection and the specific activity of the total hepatic protein counts/min per mg remained essentially unchanged Based on the above observations it could be assumed that the primary site of growth hormone action in methionine incorporation also depended on the question of the transport This possibility was not excluded but there were some facts opposing the transport hypothesis First methionine has been reported to be incorporated roughly equimolarly into both polyamines in rat liver (RAINA 1964) As shown in the table spermine activity remained unchanged after the hormone treatment If the hormone would only act on the transport of methionine into the liver an increased amount of methionine must also be available for spermine synthesis and the spermine activity is expected to increase in the same way as that of spermidine Secondly the essentially unchanged activity in the total liver protein also indicated that a specific stimulation of spermidine synthesis was involved

T a b l e 11 Incorporation of radioactivity from ^{14}C methionine into liver polyamines and total protein i n v i v o after growth hormone injection The animals were one month old male rats receiving 4 international units of growth hormone (Somacton) or solvent of the drug and 3 µC (≈ 0 62 µmole) of DL methionine - ^{14}C 3 h before analysis Three animals in each group Specific activities are presented as count /min per µmole of polyamines or mg of protein (ranges in parentheses)

Treatment	Specific activity		
	Spermidine	Spermine	Protein
Solvent	350 (310—390)	310 (250—410)	710 (700—720)
Growth hormone	670 (580—840)	270 (240—280)	750 (690—810)

Concluding remarks

There are some similarities between liver regeneration and growth hormone action on polyamine biosynthesis in rat liver. Both situations cause an increase in the concentration of liver spermidine and the mechanism by which the biosynthetic pathway of this polyamine is stimulated seems to be to some extent comparable. In both cases the formation of putrescine from its precursors was greatly enhanced while the incorporation of radioactivity from exogenous ^{14}C putrescine into spermidine was not necessarily increased. On the other hand, because of the markedly increased formation of putrescine, it seemed that the amount of endogenous putrescine being incorporated into spermidine was greater in the livers of partially hepatectomized or growth hormone-treated rats than in the controls, which was apparently reflected in the increased incorporation of label from methionine into spermidine.

Discussion

The results of the present investigation demonstrate the presence of putrescine a common diamine in the bacterial world as also occurring in rat liver tissue Although the presence of this diamine in the tissues of some mammalian species has been known for years putrescine has not really been included among the normal compounds found in fresh mammalian tissues The significance of putrescine in mammalian polyamine metabolism is still partly obscure but based on the present results it seems that this diamine serves as the natural precursor of polyamine synthesis also in mammalian tissues The putrescine concentration in rat liver is only one tenth to one third of spermidine On the other hand its conversion into polyamines is relatively rapid in mammalian tissues too indicating a rapid turnover rate of this compound as compared with the slower turnover rates of the polyamines spermidine and spermine (SIIMES 1967)

The conversion of putrescine into polyamines in the liver or other tissues is not at all the major metabolic route for intraperitoneally administered putrescine since this compound is very rapidly oxidized into carbon dioxide The location of this oxidation as well as the possible intermediates are not known

Apparently a considerable portion of hepatic putrescine is converted into polyamines in an intact rat liver Spermidine synthesis also occurs *in vitro* in liver slices and homogenates even if this reaction is possibly not as rapid as *in vivo*

The behaviour of the polyamines (RAINA 1963) and of the diamines (CALDARERA BARBIROLI and MORUZZI 1965) in the developing chick embryo correlate well with the other metabolic alterations occurring during embryonic development The behaviour of the putrescine concentration *during the postnatal growth of the rat is a more complex question* The putrescine concentration is just at its lowest during the first weeks after birth and thereafter rapidly increases A converse mode of behaviour is shown in the concentration of spermidine it is at its highest at birth and thereafter decreases (JÄNNE RAINA and SIIMES 1964) The putrescine concentration correlates to some extent with the growth phases of

the rat but this correlation is not very clear During the first weeks of postpartum life the liver of the rat does not grow as rapidly as the whole body This lag phase in liver growth lasts only one to two weeks after which the liver weight and its percentage of the total body weight rapidly increases (OLIVER BALLARD SHIELD and BENTLEY 1962 OLIVER and BLUMER 1964) The period of rapid growth is characterized by an increase in deoxyribonucleic acid concentration followed by an increase in the concentration of ribonucleic acid (OLIVER and BLUMER 1964 WINICK and NOBLE 1965) Thus the putrescine concentration is low during the lag phase and then rapidly increases reaching a maximum between the first and second month The conversion of putrescine into spermidine also increases at least *in vitro* during the first months of life And interestingly enough the activity of the S adenosylmethionine synthesizing enzyme has also been reported as increasing in the mouse and rabbit liver in a similar manner to that of spermidine synthesis from putrescine in the rat liver (HANCOCK 1966)

At first sight the behaviour of the concentration of putrescine and its conversion into spermidine are difficult to compare with the behaviour of the spermidine concentration during the postnatal development of the rat The biosynthesis of spermidine from putrescine increases while the concentration of this polyamine decreases Thus it should be assumed that the increased synthesis of spermidine from putrescine prevents a marked decrease in spermidine concentration due to increased formation of spermine from spermidine at that time (Fig 6 A SIIMES 1967)

The results of the present investigation do not absolutely illustrate the role of putrescine as acting as the *main* four-carbon precursor in the synthesis of spermidine in rat liver for other precursors have been neither excluded nor shown The incorporation of radioactivity from ^{14}C methionine into spermidine and spermine in rat tissues has been demonstrated by RAINA (1964) The idea that these compounds incorporate equimolarly together to form a spermidine molecule was supported by experiments dealing with the early action of ethionine on spermidine synthesis from putrescine Ethionine the ethyl analogue of methionine causes a deprivation of tissue adenosine triphosphate (SHULL 1962 BARTELS and HOHORST 1963) because of the formation of S adenosylethionine (MODY BULBA HOLOWECKY and STEKOL 1963 RAINA JÄNNE and SIIMES 1964 SHULL McCONOMY VOGT CASTILLO and FARBER 1966) An other early effect of ethionine is a decrease in the hepatic spermidine concentration (RAINA JÄNNE and SIIMES 1964) The

inhibition of spermidine synthesis from putrescine as shown in this investigation seems to be the result of a decrease in the substrate concentration i e S adenosylmethionine The lower level of this substrate is reflected by a decrease in the incorporation of putrescine into spermidine The very slight effects obtained by adding methionine or adenosine triphosphate to the incubation medium could depend on the slow formation of S adenosylmethionine from its precursors under these *in vitro* conditions

Compared with the developmental growth of an animal some extreme experimental conditions provide much more information on the metabo lism of polyamines in rat hepatic tissue Thus the removal of a portion of the mammalian liver provokes a series of biochemical events including changes in both putrescine and polyamine biosynthesis The morphological as well as biochemical changes taking place in regenerating liver are reviewed in detail elsewhere (HARKNESS 1957 BUCHER 1963) Some of these however will be mentioned here

The mo t rapid changes in liver chemical components occur in the area of storage products i e there is an early depletion of liver glycogen and accumulation of fats in the liver (HARKNESS 1957) The hepatic concentrations of some amino acids and related compounds, including glutamic and aspartic acids lysine and ethanolamine phosphoric ester increase rapidly after partial hepatectomy (FERRARI and HARKNESS 1954)

The most interesting and also quite rapid changes occur in the area of nucleic acid metabolism Almost immediately after partial hepatectomy there is a marked stimulation in the incorporation of labelled precursors into ribonucleic acid (BUCHER 1963 FUJIOKA KOGA and LIEBERMAN 1963 BRESNICK 1965) The incorporation of ^{14}C-orotate into hepatic ribonucleic acid begins to arise immediately after operation and reaches a maximum as early as 5 h postoperatively (FUJIOKA KOGA and LIEBER MAN 1963) On the other hand, deoxyribonucleic acid synthesis shows a rise later at about 15 to 18 h after partial hepatectomy (BUCHER 1963) The synthesis of ribonucleic acid catalyzed by ribonucleic acid polymerase is stimulated at about 6 h and reaches its maximum at 12 to 24 h after operation (BUSCH CHAMBON MANDEL and WEILL 196_ RO and BUSCH 1967)

As shown in the present investigation the accumulation of putrescine takes place almost immediately after partial hepatectomy i e it reaches a maximum of about twice the initial concentration as early as 4 h

postoperatively This accumulation is probably not a result of the diminished detoxication capacity of the remaining liver since a several fold increase *in the* incorporation of label from ^{14}C-ornithine into this diamine was observed The significance of the accumulation of putrescine is still obscure but the increased biosynthesis of putrescine from ornithine could be explained in terms of some other events taking place during the liver regeneration.

Fig 19 illustrates a possible association of putrescine and polyamine metabolism with the urea cycle and the early steps of pyrimidine biosynthesis The total urea output is somewhat lowered during the *first three* days after partial hepatectomy (HARKNESS 1957) and there are some changes in the activities of the enzymes concerning urea formation Only the activity of two enzymes however changes markedly during liver regeneration. There is a decrease in the activity of ornithine transcarbamylase (COHEN and SALLACH 1961 KIM and COHEN 1965) and a smaller decrease in the activity of arginase (RÄIHÄ JÄNNE and SUIHKONEN 1967) The other enzymes concerned urea biosynthesis in rat liver remain essentially unchanged The common precursor carbamyl phosphate does not only serve as a substrate for the urea forming system but also acts as a precursor in pyrimidine synthesis in hepatic tissue One of the first steps of pyrimidine synthesis is catalyzed by aspartate transcarbamylase In the case of rat liver the observation has been made that aspartate transcarbamylase activity increases considerably during the first days after partial hepatectomy

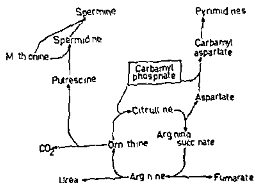


Fig 19 *Schematic representation of the postulated interrelationships between urea pyrimidine and polyamine biosynthesis in rat liver*

(CALVA and COHEN 1959 KIM and COHEN 1965 BRESNICK 1965) Thus it is possible that the amount of carbamyl phosphate available for urea formation decreases during liver regeneration because of a marked increase in aspartate transcarbamylase activity This hypothesis is supported by the finding that carbamyl phosphate synthetase activity remains quite constant during regeneration although there is a greater need for this compound in pyrimidine synthesis (KIM and COHEN 1965)

In the present study it was shown that the incorporation of radio activity from ^{14}C-ornithine into putrescine increases several fold within a few hours after partial hepatectomy Thus there are two different systems competing for the substrates of ornithine transcarbamylase one utilizing carbamyl phosphate for pyrimidine synthesis and the other using ornithine for the formation of putrescine and polyamines During active regeneration these two competing systems are considerably stimulated and the activity of ornithine transcarbamylase decreases On the other hand the maximum decrease in ornithine transcarbamylase activity occurs at about 4 to 5 days after partial hepatectomy (KIM and COHEN 1965 RÄIHÄ JÄNNE and SUIHKONEN 1067) in contrast with the peaks of aspartate transcarb amylase activity at 48 h and putrescine formation from ornithine at 8 h respectively The following explanation for this apparent discrepancy will be postulated Ornithine transcarbamylase activity might decrease in an adaptive manner because of the abolished or markedly reduced supply of the substrates carbamyl phosphate and ornithine during the first day after partial hepatectomy Assuming that the half life of ornithine transcarb amylase is like that of arginase (SCHIMKE BROWN and SMALLMAN 1063 SCHIMKE 1964) 4 to 5 days a decrease of 50 per cent in the enzyme activity should occur not until 4 to 5 days after the end of the enzyme protein synthesis If correct this hypothesis means that during liver regeneration the substrate pools carbamyl pho phate and ornithine remain essentially unchanged but that the substrates are utilized in a different manner for the urea cycle pyrimidines and polyamines respectively

The behaviour of spermidine biosynthesis from putrescine during early regeneration remains partly unexplained The incorporation of radioactivity from exogenous putrescine into spermidine is in fact decreased or remains unchanged for a few hours after partial hepatectomy On the other hand an increase in the concentration of spermidine can be seen as early as 24 h postoperatively and the synthesis of spermidine from methionine is stimulated practically immediately after operation (RAINA JÄNNE and

SIIMES 1965 1966) The incorporation of label from ornithine and arginine into putrescine is increased several fold after partial hepatectomy and the activity of hepatic spermidine derived from the above mentioned precursors also *increased* very markedly immediately after operation The formation of spermidine from ornithine and arginine occurs in all probability *via* putrescine in rat liver Thus it must be assumed that the use of exogenously administered putrescine differs from that of endogenous putrescine resulting in an apparent decrease in the incorporation of radioactivity from exogenous ^{14}C putrescine into spermidine during liver regeneration The increase in the synthesis of spermidine in regenerating liver as shown in the incorporation pattern of methionine into this polyamine (RAINA JÄNNE and SIIMES 1965 1966) could be explained in terms of increased substrate concentration of putrescine after partial hepatectomy rather than by a specific enzyme induction at this step This hypothesis is supported by the fact that also in normal liver homogenates increasing concentrations of putrescine caused a several fold increase in the incorporation of radioactivity from ^{14}C methionine into spermidine

The significance of the accumulation of spermidine and putrescine in rat liver during the early stages of regeneration is not at all clear but it could be assumed that they are in some way related to nucleic acids especially ribonucleic acid Putrescine and polyamines exert stabilizing effects on cellular polyanions in microorganisms (TABOR and TABOR 1964) In animal tissues too there are connections between these compounds and nucleic acids e g in regenerating rat liver (DYKSTRA and HERBST 1965 RAINA JÄNNE and SIIMES 1965 1966) in regard to the subcellular distribution (RAINA and TELARANTA 1967) in developing chick embryos (RAINA 1963 CALDARERA BARBIROLI and MORUZZI 1965) *etc* The simultaneous stimulation of nucleic acid synthesis and that of their stabilizers should be practical for a cell population undergoing rapid growth

Spermidine spermine and putrescine stimulate bacterial ribonucleic acid polymerase *in vitro* (KRAKOW 1963 FOX and WEISS 1964) The activity of this enzyme is also increased during the first day of regeneration (BUSCH CHAMBON MANDEL and WEILL 196_ RO and BUSCH 1967) and it is not impossible that putrescine and polyamines could affect the polymerase enzyme in a regulatory manner even in animal tissues

Based on the results of the present investigation it seems that the increase in putrescine and spermidine concentration after partial hepatectomy occurred as the result of prior stimulation of putrescine biosynthesis from

its precursors The changes in polyamine metabolism after growth hormone treatment are at least to some extent comparable with those observed in regenerating liver Among the earliest events following growth hormone treatment are the stimulation of amino acid transport into cells (RIGGS and WALKER 1960) and changes involving ribonucleic acid synthesis (TALWAR PANDA SARIN and TOLANI 1962 KORNER 1963 1964 TALWAR and GUPTA 1964 WIDNELL and TATA 1964 1966 PEGG and KORNER 1965) The growth hormone also stimulates the incorporation of amino acids into hepatic protein in hypophysectomized rats (KORNER 1959) The stimulation of protein synthesis seems to be mediated through primary stimulation of messenger ribonucleic acid synthesis (KORNER 1963)

The growth hormone also causes early stimulation of putrescine synthesis from ornithine The incorporation of label from ^{14}C-ornithine reached a maximum at 3 h after the hormone injection On the other hand it seems evident that the synthesis of spermidine from putrescine is not necessarily stimulated but that the increased activity of spermidine after the (injection of growth hormone treated rats with ^{14}C putrescine)could depend on the markedly increased putrescine uptake by the liver tissue Thus again the net increase in spermidine concentration could be the result of increased formation of endogenous putrescine from ornithine and this in turn could cause the rise in the incorporation of methionine into spermidine

The hormonal stimulation of amino acid transport into tissues as reported by RIGGS and WALKER (1960) is not excluded in the case of ornithine and methionine but the increase in the concentration of liver spermidine and the specific stimulation of spermidine synthesis from methionine as compared with spermine indicate that the effects caused by the growth hormone on polyamine metabolism, can not solely be dependent on the stimulation of the transport of radioactive precursors into the liver tissue

Thus it seems that the same step in polyamine biosynthetic pathway as in regenerating liver is stimulated after growth hormone treatment The time-course of this stimulation is very close to that previously observed in the activity of ribonucleic acid polymerase after growth hormone administ ration (WIDNELL and TATA 1966) The activity of this enzyme reached a maximum almost twice the initial value at 4 h after a single injection of growth hormone

The present investigation demonstrated three different growth processes developmental and regenerative growths and an anabolic condition caused

by the pituitary growth hormone These three situations are not directly comparable but some similarities can be seen In any case the result of the present study make it justifiable to conclude that putrescine and polyamines are in some way related to the growth processes of an animal tissue

Summary

The purpose of this study was to investigate the biosynthetic pathway involving putrescine spermidine and spermine bio ynthesis in the rat liver

The distribution of the polyamines spermidine and spermine in ani mal tissues is a relatively well documented area but there are only a few studies dealing with the occurrence of the diamine putrescine in mammalian tissues

Putrescine was shown to occur in rat liver and its concentration varied markedly in relation to age During the first two weeks of life the concentration of putrescine in the liver was low under 100 mμmoles per g wet wt increasing rapidly until the age of six weeks and thereafter again decreasing almost to the initial level The maximum concentration of liver putrescine was 270 mμmoles/g at the age of six weeks corresponding to about one third of the spermidine concentration at that time

Putrescine was rapidly converted *in vivo* into liver polyamines 4 hours after an intraperitoneal injection of putrescine-1,4 ^{14}C the activity in the liver spermidine and spermine fractions represented about 73 per cent of the activity disappeared from the hepatic putrescine fraction On the other hand the total radioactivity found in liver polyamines represented only a small percentage of that of the added dose

Intraperitoneally injected ^{14}C putrescine was very rapidly oxidized into carbon dioxide Thus the radioactivity expired as CO_2 was 37 per cent of the total dose 2 h after injection with ^{14}C putrescine Three per cent of the dose was found in the liver polyamines and 2 5 per cent in the poly amines of other tissues

Spermidine synthesis from putrescine in liver homogenates *in vitro* was also demonstrated The synthesis of spermidine from putrescine *in vitro* varied in a similar manner to that of putrescine concentration in relation to age

Administration of DL-ethionine to rats caused a several fold decrease in the incorporation of label from putrescine into spermidine within a

hours *in vivo* This inhibitory effect of ethionine was only transitory and the spermidine synthesis was almost normalized after 4-day treatment The inhibitory effect of ethionine on the spermidine synthesis in liver homogenates was slightly diminished by adding methionine or adenosine triphosphate to the incubation media

Many changes in putrescine and polyamine synthesis were observed in rat liver undergoing regeneration after partial hepatectomy The concentration of liver putrescine increased over two fold within 4 hours atfer partial hepatectomy and its biosynthesis from ornithine and arginine was greatly increased immediately after operation reaching a maximum at 8 h postoperatively The label from ornithine and arginine was also incorporated into spermidine whose activity increased several fold after partial hepatectomy too On the other hand the incorporation of radioactivity from exogenous ^{14}C putrescine into spermidine did not increase during regeneration and even decreased when a carrier free isotope was used A similar decrease in the synthesis of spermidine from putrescine was observed under *in vitro* conditions These results suggest that the increase in spermidine synthesis in regenerating rat liver is due to a prior increase in putrescine biosynthesis and accumulation

Some similarities were observed in putrescine and polyamine metabolism between rats subjecetd to partial hepatectomy or treated with the pituitary growth hormone The growth hormone caused a slight accumulation of liver spermidine at 12 h after a single hormone injection The concentrations of liver putrescine and spermine remained es entially unchanged On the other hand this hormone possessed a considerable stimulatory effect on putrescine synthesis from ^{14}C-ornithine since more than 5 fold increase in the incorporation of radioactivity from ornithine into putrescine was observed as early as 3 h after the hormone injection In addition to the synthesis of spermidine from putrescine, the uptake of putrescine possibly also increased after growth hormone treatment The radioactivity from ^{14}C methionine was incorporated into spermidine almost two times more rapidly in growth hormone-treated rats than in the control animals The specific activity of spermine and total liver protein remained essentially unchanged Hormone pretreatment did not stimulate the incorporation of label from ^{14}C-putrescine into spermidine in liver homogenates *in vitro*

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